A63

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 March 2001 (08.03.2001)

PCT

(10) International Publication Number
WO 01/15727 A2

(51) International Patent Classification[7]: A61K 39/39, A61P 31/00

(21) International Application Number: PCT/US00/23688

(22) International Filing Date: 29 August 2000 (29.08.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/152,174 2 September 1999 (02.09.1999) US

(71) Applicant *(for all designated States except US)*: **AQUILA BIOPHARMACEUTICALS INC.** [US/US]; 175 Crossing Boulevard, Suite 200, Framingham, MA 01702-4473 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **KENSIL, Charlotte, A.** [US/US]; 15 Camp Street, Milford, MA 01757 (US). **JENKINS, Sharon, A.** [US/US]; 745 Main Street, Bethlehem, NH 03574 (US).

(74) Agents: **SUPERKO, Colleen et al.**; Hale and Dorr, LLP, 60 State Street, Boston, MA 02109 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/15727 A2

(54) Title: COMPOSITIONS OF IMMUNOSTIMULATORY POLYMERS AND SAPONIN ADJUVANTS AND USES THEREOF

(57) Abstract: Vaccine compositions of immunostimulatory phosphazenes and saponin adjuvants and antigens and the use thereof for stimulating immunity are disclosed. Also described are immune adjuvant compositions comprising immunostimulatory phosphazenes and saponin adjuvants. The immune adjuvant compositions may be used to enhance cell-mediated immune response and to enhance antibody production. Also described is a method of stimulating immunity to an antigen.

COMPOSITIONS OF IMMUNOSTIMULATORY POLYMERS AND SAPONIN ADJUVANTS AND USES THEREOF

Inventors: Charlotte A. Kensil and Sharon A. Jenkins
(Attorney Docket No. 106941.182)

FIELD OF INVENTION

The present invention is in the field of immune adjuvants and vaccines. The compositions of the invention enhance the induction of a cell-mediated immune response and enhance antibody production.

BRIEF DESCRIPTION OF THE BACKGROUND ART

A wide variety of antigens stimulate the production of antibodies in animals and confer protection against subsequent infection. However, some antigens are unable to stimulate an effective immune response. The immunogenicity of such a relatively weak antigen is often enhanced by the simultaneous administration of the antigen with an adjuvant. Adjuvants are substances that are not immunogenic when administered alone, but will induce a state of mucosal and/or systemic immunity when combined with the antigen. The development of improved vaccine adjuvants for use in human biomedical applications has, therefore, become a priority of research.

Saponin adjuvants have been identified and purified from an aqueous extract of the bark of the South American tree, *Quillaja saponaria* Molina. A commercial source of heterogeneous Quillaja saponins is "Quil A" (Dalsgaard, *Acta Veterinaria Scandinavica*, 69:1 (1978)). "Quil A" may be further fractionated by HPLC. Among

the saponin HPLC peaks which are separable, the more predominant purified saponins have been identified as QS-7, QS-17, QS-18, and QS-21, also known as QA-7, QA-17, QA-18, and QA-21, respectively. These saponins have been substantially purified by various methods including high pressure liquid chromatography (HPLC), low pressure liquid silica chromatography, and hydrophilic interactive chromatography (HILIC). The substantially purified saponins have been found to be useful as immune adjuvants for enhancing immune responses in individuals. (Kensil, et al., U.S. Patent No. 5,057,540; Kensil, et al., *J. Immunol.* 148:2357 (1991); Marciani, et al., *Vaccine* 9:89 (1991).)

Recently, some synthetic polyelectrolytes of various molecular weights have been shown to have an adjuvant activity when combined with an antigen. Macromolecules bearing either positive or negative charges have displayed a similar immunostimulatory activity. The polyelectrolytes form complexes with antigens through electrostatic and hydrophobic bonds. On the other hand, uncharged polymers had no effect on the immune response unless the uncharged polymers were conjugated to the protein antigens. For example, the adjuvant activity of polyacrylic acid (PAA), copolymers of acrylic acid and N-vinylpyrrolidone (CP-AAVPD), poly-2-methyl-5-vinyl pyridine (PMVP), poly-4-vinyl-N-ethylpyridinium bromide (PVP-R_2) and similar compounds, when conjugated to an antigen, has been studied by Petrov, et al., *Journal Vses. Khim. Ob-va im. D. I. Mendeleeva* 33:22-42 (1988). The immunomodulatory effect of polyelectrolyte complexes containing many of these same polyelectrolytes has been more recently reviewed by Petrov, et al., *Sov. Med. Rev. D.*

Immunol., 4:1-113 (1992).

One polyelectrolyte, polyphosphazene, has been shown to be useful both as a soluble adjuvant and as an encapsulation vehicle for antigen. Antigens may be mixed with the soluble polyphosphazene and injected directly into an animal for parenteral immunization. (Payne, et al., *Pharm. Biotechnol.*, 6:473-493 (1995).)

Because polymers can entrap antigens, microencapsulation has been applied to the injection of pharmaceuticals to give a controlled release, *i.e.*, poly-D,L-lactidecoglycolide (PLGA). In a similar fashion, polyphosphazene microspheres, because of their ability to cross-link and their water-solublity characteristics, have become an interesting potential vaccine delivery vehicle. (Andrianov, et al., U.S. Patent No. 5,579,777.) A water-soluble polyphosphazene and antigen solution may be formulated into hydrogel microspheres by ionically cross-linking the carboxyl groups with divalent cations, and then used for parenteral or mucosal administration. (Payne, et al., *supra*.)

SUMMARY OF THE INVENTION

Saponin and polymeric adjuvants have each proved to be effective immune stimulators in eliciting certain types of immune responses. However, there is a typical maximum level of stimulation from a single adjuvant with a given antigen. It would be useful to further optimize the maximum level of immune stimulation. Optimization of an adjuvant effect with a single adjuvant and a single antigen is usually defined by a dose response curve of adjuvant; a maximum or "plateau" level

of immune response is achieved at an optimum dose of adjuvant. Further increases in adjuvant effect are not expected from these adjuvants alone by going to higher doses. However there is a need for optimized adjuvant formulations that may drive the immune stimulation to a higher maximum level. Accordingly, the present invention provides an effective immune adjuvant composition of one or more saponin adjuvant and an immunostimulatory polymeric adjuvant that provides for a higher maximum level of immune stimulation. An adjuvant may provide immune stimulation that is delayed (*i.e.*, appearing after multiple immunizations). There is a need for optimized adjuvant formulations that provide an earlier immune stimulation. Accordingly, the present invention provides an immune adjuvant composition that also raises an enhanced immune response at an earlier time in an immunization schedule. An adjuvant may be required in large quantities for optimal immune response. There is a need for optimized adjuvant formulations that allow the use of less adjuvant, but with equivalent immune stimulation to higher adjuvant doses, used alone. Accordingly, the present invention provides an immune adjuvant composition that provides enhanced immune response (antibody and CTL) compared to low doses of the adjuvants alone.

DESCRIPTION OF THE FIGURES

Figure 1 shows the chemical structure of the saponin adjuvant QS-21.

Figure 2 depicts a graph showing the enhanced induction of an antibody response of mice having received immunizations with ovalbumin and various

adjuvant formulations. C57BL/6 mice were immunized subcutaneously with ovalbumin (25 μg) and various adjuvant formulations consisting either of QS-21 at suboptimal (1.25 μg) or optimal (10 μg) doses, polyphosphazene at suboptimal (10 μg) or optimal (100 μg) doses, as well as combinations of the two adjuvants. Mice received immunizations at days 0, 14, and 28. Sera were collected for analysis of anti-ovalbumin IgG at day 42.

Figure 3 provides a graph showing the enhanced induction of a cell-mediated immune response by the QS-21 and polyphosphazene combination, as evidenced by the CTL induction. C57BL/6 mice were immunized subcutaneously with ovalbumin (25 μg) and various adjuvant formulations consisting either of QS-21 at suboptimal (1.25 μg) or optimal (10 μg) doses, polyphosphazene at suboptimal (10 μg) or optimal (100 μg) doses, as well as combinations of the two adjuvants. Mice received immunizations at days 0, 14, and 28. Spleens were collected for analysis of antigen-specific cytotoxic T lymphocyte response at day 42. Splenocytes were stimulated *in vitro* for 6 days with mitomycin C-treated E.G7-OVA cells. The stimulated splenocytes were used in a standard CTL assay using ^{51}Cr-loaded E.G7-OVA cells as targets.

Figure 4 provides a graph of the induced IgG antibody production. The data show the influenza specific serum IgG response as measured by ELISA. Balb/C mice were subcutaneously immunized with split inactivated influenza virus strain X-31 vaccine (5 μg) and various adjuvant formulations consisting either of QS-21 at suboptimal (2 μg) or optimal (20 μg) doses, polyphosphazene at suboptimal (10 μg)

or optimal (100 μg) doses, as well as combinations of the two adjuvants. Mice received only a single immunization on day 0. Sera were collected for analysis of anti-influenza IgG at days 21 and 42.

Figure 5 shows a graph of enhanced IgG2a antibody production, a response that is influenced by Th 1 cytokines. These data show the influenza specific serum IgG2a response, as measured by ELISA, from mice having received immunizations with influenza vaccine and various adjuvant formulations. Balb/C mice were subcutaneously immunized with split inactivated influenza virus strain X-31 vaccine (5 μg) and various adjuvant formulations consisting either of QS-21 at suboptimal (2 μg) or optimal (20 μg) doses, polyphosphazene at suboptimal (10 μg) or optimal (100 μg) doses, as well as combinations of the two adjuvants. Mice received only a single immunization at day 0. Sera were collected for analysis of anti-influenza IgG2a at days 21 and 42.

Figure 6 shows a graph of the enhanced IgG1 antibody production. The data show the influenza specific serum IgG1 response as measured by ELISA. Balb/C mice were subcutaneously immunized with split inactivated influenza virus strain X-31 vaccine (5 μg) and various adjuvant formulations consisting either of QS-21 at suboptimal (2 μg) or optimal (20 μg) doses, polyphosphazene at suboptimal (10 μg) or optimal (100 μg) doses, as well as combinations of the two adjuvants. Mice received only a single immunization on day 0. Sera were collected for analysis of anti-influenza IgG1 at days 21 and 42.

Figure 7 depicts the functional antibody responses as measured by

hemagglutination inhibition assay. Balb/C mice were subcutaneously immunized with influenza virus (5 μg) and various adjuvant formulations consisting either of QS-21 at suboptimal (2 μg) or optimal (20 μg) doses, polyphosphazene at suboptimal (10 μg) or optimal (100 μg) doses, as well as combinations of the two adjuvants. Mice received only a single immunization on day 0. Sera were collected for analysis of hemagglutination inhibition titers at days 21 and 42.

Figure 8 provides a graph of induced IgG1 antibody production for mice having received immunizations with polysaccharide and various adjuvant formulations. Balb/C mice were immunized subcutaneously with 0.5 μg Type 14 *S. pneumonia* capsular polysaccharide (antigen) alone or with adjuvant formulations consisting of QS-21 at suboptimal (1.25 μg) or optimal (10 μg) doses, polyphosphazene (100 μg), and combinations of the two adjuvants. Sera were collected and serum titers to polysaccharide were determined by ELISA on day 21 (mice immunized once, on day 0) or on day 42 (mice immunized twice, on days 0 and 28).

Figure 9 depicts the enhanced IgG2a response for mice having received immunizations with polysaccharide and various adjuvant formulations. Balb/C mice were immunized subcutaneously with 0.5 μg Type 14 *S. pneumonia* capsular polysaccharide (antigen) alone or with adjuvant formulations consisting of QS-21 at suboptimal (1.25 μg) or optimal (10 μg) doses, polyphosphazene (100 μg), and combinations of the two adjuvants. Sera were collected and serum titers to polysaccharide were determined by ELISA on day 21 (mice immunized once, on day

0) or on day 42 (mice immunized twice, on days 0 and 28).

Figure 10 shows a graph of the induced IgG3 antibody production for mice having received immunizations with polysaccharide and various adjuvant formulations. Balb/C mice were immunized subcutaneously with 0.5 μg Type 14 *S. pneumonia* capsular polysaccharide (antigen) alone or with adjuvant formulations consisting of QS-21 at suboptimal (1.25 μg) or optimal (10 μg) doses, polyphosphazene (100 μg), and combinations of the two adjuvants. Sera were collected and serum titers to polysaccharide were determined by ELISA on day 21 (mice immunized once, on day 0) or on day 42 (mice immunized twice, on days 0 and 28).

DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

Since rapid development of Th 1 and Th 2 immunity plays an important role in the protective response to infection with certain microbial agents, a need exists to characterize other novel adjuvant compositions that may safely induce these responses and may potentially be incorporated in future human vaccines. Surprisingly, a combination of a synthetic, water-soluble, immunostimulatory phosphazene and a saponin adjuvant was found to be a powerful stimulator of immunity compared to either adjuvant alone. These results establish that an immune adjuvant composition comprising an immunostimulatory polymeric adjuvant and a saponin adjuvant is a candidate adjuvant for vaccines to induce immunity.

In a first aspect of the invention, an immune adjuvant composition comprising one or more saponin adjuvants and an immunostimulatory polymeric adjuvant may be administered.

In one preferred embodiment, the immunostimulatory polymeric adjuvant is a synthetic, water-soluble, immunostimulatory polyelectrolyte. In a preferred embodiment, the synthetic, water-soluble, immunostimulatory polyelectrolyte is a polyphosphazene. In a further embodiment, both the phosphazene and the saponin are biodegradable and exhibit minimal toxicity when administered to individuals, which may be humans or other animals.

In another embodiment, the immunostimulatory polymeric adjuvant in the immune adjuvant composition is a polyorganophosphazene with an ionized or ionizable pendant group that contains, for example, carboxylic acid or hydroxyl moieties. In such an instance, the preferred immunostimulatory polymeric adjuvant is poly[di(carboxylatophenoxy)phosphazene] or "PCPP." Alternatively, another embodiment is directed towards the immunostimulatory polymeric adjuvant polyorganophosphazene with (i) an ionized or ionizable pendant group that contains carboxylic acid or hydroxyl moieties and (ii) a pendant group that is susceptible to hydrolysis under the conditions of use, to impart biodegradability to the polymer and wherein the pendant group that is susceptible to hydrolysis is selected from chlorine, amino acid, amino acid ester, imidazole, glycerol, and glucosyl. Two examples of polyphosphazenes that are useful as immune adjuvants within this genus are poly[di(carboxylatophenoxy)phosphazene-co-di(glycinato)phosphazene-

co(carboxylatophenoxy)(glycinato)phosphazene] and poly [di(carboxylatophenoxy)phosphazene-co-di(chloro)phosphazene-co-(carboxylatophenoxy)-(chloro)phosphazene].

The saponin adjuvant in the immune adjuvant composition may comprise an unpurified, partially purified, or substantially purified saponin. Preferably, the saponin adjuvant in the immune adjuvant composition is from *Quillaja saponaria* Molina. More preferably, the saponin adjuvant may encompass a partially purified saponin-enriched extract "Quil-A." (Dalsgaard, *supra.*) Most preferably, the saponin adjuvant is a substantially purified saponin, QS-7, QS-17, QS-18, or QS-21, also known as QA-7, QA-17, QA-18, or QA-21, respectively. QS-21 is the most preferred saponin adjuvant in the immune adjuvant composition. In a further preferred embodiment, the saponin adjuvant may cover a semisynthetic saponin adjuvant. In another preferred embodiment, the saponin adjuvant may cover a chemically modified saponin adjuvant or a fraction thereof obtainable from an unpurified *Quillaja saponaria* extract, wherein the chemical modification of the saponin or fraction thereof consists of conjugation of the saponin glucuronic acid carboxyl to an antigen.

A second aspect of the invention provides novel vaccine compositions comprising an immunostimulatory polymeric adjuvant, one or more saponin adjuvants, and an antigen. Preferably, the vaccine composition stimulates Th 1 and Th 2 immunity. More preferably, the vaccine composition enhances antibody production to an antigen and enhances induction of a cell-mediated immune response to an antigen. In another embodiment, the vaccine composition, most preferably, will

synergistically enhance a cell-mediated immune response to an antigen.

The saponin adjuvant in the vaccine composition may be an unpurified, partially purified, or substantially purified saponin. Preferably, the saponin adjuvant in the vaccine composition is from the tree *Quillaja saponaria* Molina. More preferably, the saponin adjuvant may encompass a partially purified saponin-enriched extract "Quil-A." (Dalsgaard, *supra*.) Most preferably, the saponin adjuvant is a substantially purified saponin, QS-7, QS-17, QS-18, or QS-21, also known as QA-7, QA-17, QA-18, or QA-21, respectively. QS-21 is the most preferred saponin adjuvant in the immune adjuvant composition. In a further preferred embodiment, the saponin adjuvant may cover a semisynthetic saponin adjuvant. In another preferred embodiment, the saponin adjuvant may cover a chemically modified saponin adjuvant or a fraction thereof obtainable from an unpurified *Quillaja saponaria* extract, wherein the chemical modification of the saponin or fraction thereof consists of conjugation of the saponin glucuronic acid carboxyl to an antigen.

In one preferred embodiment, the immunostimulatory polymeric adjuvant is a synthetic, water-soluble, immunostimulatory polyelectrolyte. In a preferred embodiment, the synthetic, water-soluble, immunostimulatory polyelectrolyte is a polyphosphazene. In a further embodiment, both the phosphazene and the saponin are biodegradable and exhibit minimal toxicity when administered to individuals, which may be humans or other animals. More preferably, such a vaccine composition may increase an immune response to an antigen in an individual to which the antigen is administered.

In a further embodiment, the immunostimulatory polymer in the vaccine composition is a polyorganophosphazene with an ionized or ionizable pendant group that contains, for example, carboxylic acid or hydroxyl moieties. In such an instance, the preferred immunostimulatory polymeric adjuvant is poly[di(carboxylatophenoxy)phosphazene] or "PCPP." Alternatively, another embodiment is directed towards the immunostimulatory polymeric adjuvant polyorganophosphazene with (i) an ionized or ionizable pendant group that contains carboxylic acid or hydroxyl moieties and (ii) a pendant group that is susceptible to hydrolysis under the conditions of use, to impart biodegradability to the polymer and wherein the pendant group that is susceptible to hydrolysis is selected from chlorine, amino acid, amino acid ester, imidazole, glycerol, and glucosyl. Two examples of polyphosphazenes that are useful as immune adjuvants within this genus are poly[di(carboxylatophenoxy)phosphazene-co-di(glycinato)phosphazene-co(carboxylatophenoxy)(glycinato)phosphazene] and poly [di(carboxylatophenoxy)phosphazene-co-di(chloro)phosphazene-co-(carboxylatophenoxy)-(chloro)phosphazene].

A third aspect of the invention is a method for stimulating the immune response to an antigen in an individual to which the antigen is administered comprising administering an effective amount of a vaccine composition comprising one or more saponin adjuvants, an immunostimulatory polymeric adjuvant, and an antigen.

Other useful methods for using the disclosed immune adjuvant compositions

that fall within the scope of the invention include enhancing antibody production to an antigen and enhancing induction of a cell-mediated immune response to an antigen. Accordingly, a fourth aspect of the invention is a method for enhancing antibody production to an antigen in an individual comprising administering an effective amount of an immune adjuvant composition comprising one or more saponin adjuvant and an immunostimulatory polymeric adjuvant. A fifth aspect encompasses a method for enhancing the induction of a cell-mediated immune response to an antigen in an individual comprising administering an effective amount of an immune adjuvant composition comparing one or more saponin adjuvants and an immunostimulatory polymeric adjuvant.

The saponin adjuvant in the immune adjuvant composition according to these method may be an unpurified, partially purified, or substantially purified saponin. Preferably, the saponin adjuvant in the immune adjuvant composition is from *Quillaja saponaria* Molina. More preferably, the saponin adjuvant may encompass a partially purified saponin-enriched extract "Quil-A." (Dalsgaard, *supra.*) Most preferably, the saponin adjuvant is a substantially purified saponin, QS-7, QS-17, QS-18, or QS-21, also known as QA-7, QA-17, QA-18, or QA-21, respectively. QS-21 is the most preferred saponin adjuvant in the method using the immune adjuvant composition. In a further preferred embodiment, the saponin adjuvant may cover a semisynthetic saponin adjuvant. In another preferred embodiment, the saponin adjuvant may cover a chemically modified saponin adjuvant or a fraction thereof obtainable from an unpurified *Quillaja saponaria* extract, wherein the chemical modification of the saponin

or fraction thereof consists of conjugation of the saponin glucuronic acid carboxyl to an antigen.

In one preferred embodiment, the immunostimulatory polymeric adjuvant is a synthetic, water-soluble, immunostimulatory polyelectrolyte. In a preferred embodiment, the synthetic, water-soluble, immunostimulatory polyelectrolyte is a polyphosphazene. In a further embodiment, both the phosphazene and the saponin are biodegradable and exhibit minimal toxicity when administered to individuals, which may be humans or other animals.

In another embodiment of these methods, the immunostimulatory polymeric adjuvant in the immune adjuvant composition administered in such method is a phosphazene, preferably a polyorganophosphazene with (i) ionized or ionizable pendant groups that contain, for example, carboxylic acid or hydroxyl moieties, and (ii) pendant groups that are susceptible to hydrolysis under the conditions of use, to impart biodegradability to the polymer. Suitable hydrolyzable groups include, for example, chlorine, amino acid, amino acid ester, imidazole, glycerol, and glucosyl. Two examples of polyphosphazenes that are useful as immunoadjuvants are poly[di(carboxylatophenoxy)phosphazene-co-di(glycinato)phosphazene-co(carboxylatophenoxy)(glycinato)phosphazene] and poly [di(carboxylatophenoxy)phosphazene-co-di(chloro)phosphazene-co-(carboxylatophenoxy)-(chloro)phosphazene].

The details of the preferred embodiments of the invention are more fully described below.

The saponins of the present invention may be unpurified, partially purified, or substantially purified saponins from any source. The saponins may obtained from the tree *Quillaja saponaria* Molina. A commercial source of partially purified heterogeneous Quillaja saponins is "Quil A". (Dalsgaard, *supra.*) "Quil A" may be further fractionated by HPLC. Among the HPLC peaks which are separable, the more predominant purified saponins have been identified as QS-7, QS-17, QS-18, and QS-21, also known as QA-7, QA-17, QA-18, and QA-21, respectively. These saponins have been substantially purified by various methods including high pressure liquid chromatography (HPLC), low pressure liquid silica chromatography, and hydrophilic interactive chromatography (HILIC).

The term "saponin" as used herein includes glycosidic triterpenoid compounds which produce foam in aqueous solution, have hemolytic activity in most cases, and possess immune adjuvant activity. The term encompasses the saponin *per se*, as well as natural and pharmaceutically acceptable salts thereof and pharmaceutically acceptable derivatives thereof. The term "saponin" also encompasses biologically active fragments thereof, as well as semisynthetic saponins.

As described in Kensil, et al., U.S. Patent No. 5,057,540, the contents of which are fully incorporated by reference herein, the adjuvant activity of such saponins may be determined by any of a number of methods known to those of ordinary skill in the art. The increase in antibody titer of antibody against specific antigen upon administration of an adjuvant may be used as a criteria for adjuvant activity and determined by any of several methods. (Dalsgaard, *Acta Verterinia Scandinavica*, 69:1

(1978); Bomford, *Int. Archs. Allergy Appl. Immun.* 77:409 (1985).) Briefly, one such test involves injecting CD-1 mice intradermally with an antigen (for instance, *i.e.*, bovine serum albumin, BSA) mixed with varying amounts of the potential adjuvant. Sera are harvested from the mice two weeks later and tested by ELISA for anti-BSA antibody. Another such test involves injecting inbred mice such as C57BL/6 or Balb/c by subcutaneous route with a protein antigen such as ovalbumin ("OVA"), an inactivated viral vaccine such as a flu vaccine, or a polysaccharide antigen such as pneumococcal polysaccharide, mixed with the potential adjuvant. Sera harvested from the mice after one, wo or three immunizations could be harvested and tested by ELISA for antigen-specific antibody (total immunoglobulin) or for specific mouse IgG subclasses such as IgG1 or IgG2a. Another such test involves injecting C57BL/6 mice with OVA, harvesting spleens after one, two, or three immunizations, stimulating splenocytes with antigen, and then assaying for cytolytic T lymphocyte activity or killing of OVA-peptide-expressing target cells. Alternative, a proliferative response could be measured in an *in vitro* assay by measuring the uptake of ^{3}H-thymidine by antigen-stimulated splenocytes obtained from immunized animals.

"QS-21" as used herein designates the mixture of components QS-21V1 and QS-21V2 which appear as a single peak on reverse phase HPLC on Vydac C4 (5 µm particle size, 300Å pore, 4.6 mm ID x 25 cm length) in 40 mM acetic acid in methanol/water (58/42, v/v). The component fractions are referred to specifically as QS-21V1 and QS-21V2 when describing experiments performed on the further purified components. The chemical structure of QS-21 is shown in Figure 1.

According to Kensil, et al., U.S. Patent No. 5,583,112, the contents of which are fully incorporated by reference herein, the carboxyl group on the glucuronic acid of *Quillaja saponaria* Molina can be conjugated to a protein, a peptide, or a small molecule containing a primary amine. Thus, the present invention relates to a chemically modified saponin adjuvant or a fraction thereof obtainable from a crude *Quillaja saponaria* Molina extract, wherein the chemically modified saponin or fraction thereof comprises at least one of QS-17, QS-18, QS-21, QS-21V1, and QS-21V2, and wherein the modified saponin retains adjuvant activity.

The term "semisynthetic" as used herein means a chemical modification of a naturally occurring compound where the compound has been modified by one or more synthetic or degradative reactions to form a compound that does not normally occur in nature. The starting materials for this compound may be purified or unpurified naturally occurring compound.

The present invention may also employ modified or semisynthetic saponins that retain adjuvant activity when administered to an individual, which may be a human or another animal. It may employ unpurified, partially purified, or substantially purified saponins.

The term "partially purified" means saponins partially separated from compounds normally associated with the saponins in its natural state.

The term "substantially purified" as used herein means substantially free from compounds normally associated with the saponin in its natural state and exhibiting constant and reproducible chromatographic response, elution profiles, and biologic

activity. The term "substantially purified" is not meant to exclude artificial or synthetic mixtures of the saponin with other compounds.

The present invention may also employ immunostimulatory saponins isolated from other plant species. For example, a saponin from *Doliches lablab* has been shown to be useful as an adjuvant [Katayan, et al., *Vaccine* 17:2733 (1999)].

The term "immunostimulatory polymeric adjuvant" or "immunostimulatory polymer" as used herein means long chained polymers that act as immune adjuvants by increasing the immune response to an antigen in an individual to which the polymeric adjuvant and the antigen are administered.

The term "polyelectrolyte" as used herein means polymers that contain ionized or ionizable pendant groups that render the polymer anionic, cationic, or amphophilic. The ionic groups can be in the form of a salt, or alternatively, an acid or base that is or can be at least partially dissociated. The polyelectrolyte can also contain non-ionic side groups.

The term "polyphosphazene" as used herein means long chained polymers of phosphazenes with backbones consisting of repeating units that have immune adjuvant properties.

The term "amino acid," as used herein, refers to both natural and synthetic amino acids, and includes, but is not limited to alanyl, valinyl, leucinyl, isoleucinyl, prolinyl, phenylalaninyl, tryptophanyl, methioninyl, glycinyl, serinyl, threoninyl, cysteinyl, tyrosinyl, asparaginyl, glutaminyl, asparatoyl, glutaoyl, lysinyl, argininyl, and histidinyl. The term "amino acid ester" refers to the aliphatic, aryl, or heteroaromatic carboxylic acid ester of a natural or synthetic amino acid.

The term "alkyl," as used herein, means a saturated straight, branched, or cyclic hydrocarbon, or a combination thereof, typically of C_1 to C_{20}, and specifically includes methyl, ethyl, propyl, isopropyl, butyl, isobutyl, t-butyl, pentyl, cyclopentyl, isopentyl, neopentyl, hexyl, isohexyl, cyclohexyl, 3-methylpentyl, 2,2-dimethylbutyl, 2,3-dimethylbutyl, heptyl, octyl, nonyl, and decyl.

The term "(alkyl or dialkyl)amino" means an amino group that has one or two alkyl substituents, respectively.

The terms "alkenyl" and "alkynyl", as used herein, means a C_2 to C_{20} straight or branched hydrocarbon with at least one double or triple bond, respectively. The term aryl, as used herein, refers to phenyl or substituted phenyl, wherein the substituent is halo, alkyl, alkoxy, alkylthio, haloalkyl, hydroxyalkyl, alkoxyalkyl, methylenedioxy, cyano, C(O)(lower alkyl), —CO_2H, — SO_3H, —PO_3H, —CO_2alkyl, amide, amino, alkylamino and dialkylamino, and wherein the aryl group may have up to 3 substituents.

The term "aliphatic" refers to hydrocarbon, typically of C_1 to C_{20}, that may contain one or a combination of alkyl, alkenyl, or alkynyl moieties, and which may be

straight, branched, or cyclic, or a combination thereof.

The term "halo," as used herein, includes fluoro, chloro, bromo, and iodo.

The term "aralkyl" refers to an aryl group with an alkyl substituent.

The term "alkaryl" refers to an alkyl group that has an aryl substituent, including benzyl, substituted benzyl, phenethyl or substituted phenethyl, wherein the substituents are as defined above for aryl groups.

The term "heteroaryl" or "heteroaromatic," as used herein, refers to an aromatic moiety that includes at least one sulfur, oxygen, or nitrogen in the aromatic ring, and that may be optionally substituted as described above for aryl groups. Nonlimiting examples are furyl, pyridyl, pyrimidyl, thienyl, isothiazolyl, imidazolyl, tetrazolyl, pyrazinyl, benzofuranyl, benzothiophenyl, quinolyl, isoquinolyl, benzothienyl, isobenzofuryl, phyrazolyl, indolyl, isoindolyl, benzimidazolyl, purinyl, carbozolyl, oxaxolyl, thiazolyl, isothiazolyl, 1,2,4thiadiazolyl, isooxazolyl, pyrrolyl, pyrazolyl, quinazolinyl, phridazinyl, pyrazinyl, cinnolinly, phthalzinyl, quinoxalinyl, xanthinyl, hypoxanthinyl, pteridinyl, 5-azacytidinyl, 5-azauracilyl, triazolopyridinyl, imidazol-opyridinyl, pyrrolopyrimidinyl, and pyrazolopyrimidinyl.

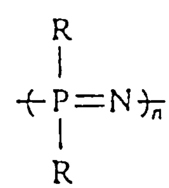
The term "pharmaceutically acceptable cation" refers to an organic or inorganic moiety that carries a positive charge and that may be administered as a counteraction in a phosphazene polyelectrolyte.

The term "heteroalkyl," as used herein, refers to an alkyl group that includes a heteroatom such as oxygen, sulfur, or nitrogen (with valence completed by hydrogen or oxygen) in the carbon chain or terminating the carbon chain.

A synthetic polymer is provided for use as an immune adjuvant. The immunostimulatory polymer is a preferably a polyphosphazene that is at least partially soluble in water (typically to an extent of at least 0.001% by weight), an aqueous buffered salt solution, or aqueous alcohol solution. The polyphosphazene preferably contains charged side groups either in the form of an acid or base that is in equilibrium with its counter ion, or in the form of an ionic salt thereof.

The polymer is preferably biodegradable and exhibits minimal toxicity when administered to individuals, which may be humans or other animals.

Polyphosphazenes are polymers with backbones consisting of alternating phosphorus and nitrogen, separated by alternating single and double bonds. Each phosphorous atom is covalently bonded to two pendant groups ("R"). The repeat unit in polyphosphazenes has the following general formula:



wherein n is an integer.

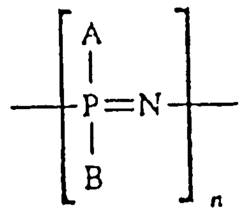
The substituent ("R") may be any of a wide variety of moieties that may vary within the polymer, including but not limited to aliphatic, aryl, aralkyl, alkaryl, carboxylic acid, heteroaromatic, carbohydrates, including glucose, heteroalkyl, halogen, (aliphatic)amino including alkylaminoheteroaralkyl, di(aliphatic)amino- including dialkylamino-, arylamino-, diarylamino-, alkylarylamino-, -oxyraryl

including but not limited to -oxyphenylCO_2H, -oxyphenylSO_3H, -oxyphenylhydroxyl and -oxyphenylPO_3H; -oxyaliphatic including -oxyalkyl, -oxy(aliphatic)CO_2H -oxy(aliphatic)SO_3H, -oxy(aliphatic)PO_3H, and -oxy(aliphatic)hydroxyl, including oxy(alkyl)hydroxyl; -oxyalkaryl, -oxyaralkyl, -thioaryl, thioaliphatic including -thioalkyl, -thioalkaryl, thioaralkyl, —NHC(O))-(aryl or aliphatic), —O—[$(CH_2)x$O]y—CH)—O—[$(CH_2)x$O]$y$$(CH_2)xNH(CH_2)x$$SO_3H$, and —O—[$(CH_2)x$O]y-(aryl or aliphatic), wherein x is 1-8 and y is an integer of 1 to 20. The groups may be bonded to the phosphorous atom through, for example, an oxygen, sulfur, nitrogen, or carbon atom.

In general, when the polyphosphazene has more than one type of pendant group, the groups will vary randomly throughout the polymer, and the polyphosphazene is thus a random copolymer. Phosphorous may be bound to two like groups, or two different groups. Polyphosphazenes with two or more types of pendant groups may be produced by reacting poly(dichlorophosphazene) with the desired nucleophile or nucleophiles in a desired ratio. The resulting ratio of pendant groups in the polyphosphazene will be determined by a number of factors, including the ratio of starting materials used to produce the polymer, the temperature at which the nucleophilic substitution reaction is carried out, and the solvent system used. While it is very difficult to determine the exact substitution pattern of the groups in the resulting polymer, the ratio of groups in the polymer may be easily determined by one skilled in the art.

In one embodiment, the immune adjuvant composition comprises a

biodegradable polyphosphazene of the formula:



wherein A and B may vary independently in the polymer, and may be:

(i) a group that is susceptible to hydrolysis under the conditions of use, including but not limited to chlorine, amino acid, amino acid ester (bound through the amino group), imidazole, glycerol, or glucosyl; or

(ii) a group that is not susceptible to hydrolysis under the conditions of use, including, but not limited to an aliphatic, aryl, aralkyl, alkaryl, carboxylic acid, heteroaromatic, heteroalkyl, (aliphatic(amino- including alkylamino-, heteroaralkyl, di(aliphatic)amino including dialkylamino-, arylamino-, diarylamino-, alkylarylamino-, oxyaryl including but not limited to -oxyphenylCO_2H, -oxyphenyl SO_3H, -oxyphenylhydroxyl and oxyphenylPO_3H; -oxyaliphatic including -oxyalkly, -oxy(aliphatic CO_2H, -oxy(aliphatic) SO_3H, -oxy(aliphatic PO_3H, and -oxy(aliphatic)hydroxyl, including -oxy(alkyl)hydroxyl; -oxyalkaryl, -oxyaralkyl, -thioaryl, -thioaliphatic including -thioalkyl, -thioalkaryl, or -thioaralkyl;

wherein the polymer contains at least one percent or more, preferably 10 percent or more, and more preferably 80 to 90 percent or more, but less than 100% of repeating units that are not susceptible to hydrolysis under the conditions of

use,

and wherein n is an integer of 4 or more, and preferably between 10 and 20,000 to 300,000.

It should be understood that certain groups, such as heteroaromatic groups other than imidazole, hydrolyze at an extremely slow rate under neutral aqueous conditions, such as that found in the blood, and therefore are typically considered nonhydrolyzable groups for purposes herein. However, under certain conditions, for example, low pH, as found, for example, in the stomach, the rate of hydrolysis of normally nonhydrolyzable groups (such as heteroaromatics other than imidazole) may increase to the point that the biodegradation properties of the polymer may be affected. One of ordinary skill in the art using well known techniques may easily determine whether pendant groups hydrolyze at a significant rate under the conditions of use. One of ordinary skill in the art may also determine the rate of hydrolysis of the polyphosphazenes of diverse structures as described herein, and will be able to select that polyphosphazene that provides the desired biodegradation profile for the targeted use.

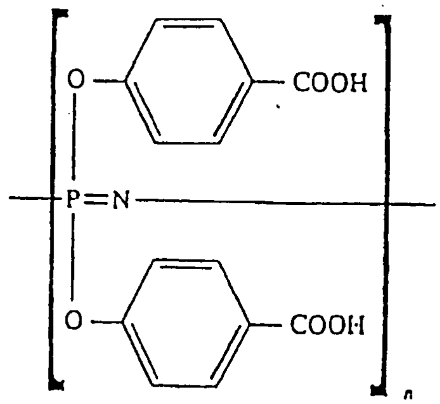
The degree of hydrolytic degradability of the polymer will be a function of the percentage of pendant groups susceptible to hydrolysis and the rate of hydrolysis of the hydrolyzable groups. The hydrolyzable groups are replaced by hydroxyl groups in aqueous environments to provide P—OH bonds that impart hydrolytic instability to the polymer.

In other embodiments, the immune adjuvant comprises: (i) a

nonbiodegradable polyphosphazene wherein none, or virtually none, of the pendant groups in the polymer are susceptible to hydrolysis under the conditions of use, or (ii) a completely biodegradable polyphosphazene wherein all of the groups are susceptible to hydrolysis under the conditions of use (for example, poly[di(glycinato)phosphazene]).

Phosphazene polyelectrolytes are defined herein as polyphosphazenes that contain ionized or ionizable pendant groups that render the polyphosphazene anionic, cationic or amphophilic. The ionic groups may be in the form of a salt, or, alternatively, an acid or base that is or may be at least partially dissociated. Any pharmaceutically acceptable monovalent cation may be used as counterion of the salt, including but not limited to sodium, potassium, and ammonium. The phosphazene polyelectrolytes may also be biodegradable or nonbiodegradable under the conditions of use. The ionized or ionizable pendant groups are preferably not susceptible to hydrolysis under the conditions of use.

A preferred polyorganophosphazene immune adjuvant contains pendant groups that include carboxylic acid, sulfonic acid, or hydroxyl moieties. While the acidic groups are usually on nonhydrolyzable pendant groups, they may alternatively, or in combination, also be positioned on hydrolyzable groups. An example of a phosphazene polyelectrolyte having carboxylic acid groups as side chains is shown in the following formula:



wherein n is an integer, preferably an integer between 10 and 10,000 to 300,000. This polymer has the chemical name poly[di(carboxylatophenoxy)phosphazene] or, alternatively, poly[bis(carboxylatophenoxy)phosphazene] (PCPP).

The polyorganophosphazene is preferably biodegradable to prevent eventual deposition and accumulation of polymer molecules at distant sites in the body, such as the spleen. The term "biodegradable," as used herein, means a polymer that degrades within a period that is acceptable in the desired application, typically less than about five years and most preferably less than about one year, once exposed to a physiological solution of pH 6-8 at a temperature of approximately 25°C-37°C.

Most preferably, the immunostimulatory polymeric is a polyorganophosphazene that includes pendant groups that include carboxylic acid moieties that do not hydrolyze under the conditions of use and pendant groups that are susceptible to hydrolysis under the conditions of use. Examples of preferred phosphazene polyelectrolytes with hydrolysis-sensitive groups are poly[-di(carboxylatophenoxy)phosphazene-co-di(aminoacid)phosphazene-co)carboxylatophenoxy)(amino acid)phosphazene], specifically including poly[di(carboxylatophenoxy)phosphazene-co-di(glycinato)phosphazene-co(carboxylatophenoxy)(glycinato)phosphazene], and

poly[di(carboxylatophenoxy(phosphazene-co-di(chloro)phosphazene-co-(carboxylatophenoxy)(chloro)phosphazene].

The toxicity of the polyphosphazene may be determined using cell culture experiments well known to those skilled in the art. For example, toxicity of PCPP may be determined in cell culture by coating cell culture dishes with the PCPP. Chicken embryo fibroblasts are then seeded onto the coated petri dishes. Three days after seeding the chicken embryo fibroblasts, the cells are inspected and cell morphology observed. Flattened cells and spindle formulation indicates non-toxicity. Under phase contrast microscopy, the presence of mitotic figures provides evidence of the non-toxicity of PCPP to replicating cells.

Crosslinked polyphosphazenes for use as immunoadjuvants may be prepared by combining a phosphazene polyelectrolyte with a metal multivalent cation such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt, nickel, or cadmium.

The term "immune adjuvant" as used herein refers to compounds which, when administered to an individual, increase the immune response to an antigen in the individual to which the antigen is administered. Preferably, such individuals are mammals, and more preferably, the mammals are humans. However, the invention is not intended to be so limiting. Any animals that may experience the beneficial effects of the vaccines of the invention are within the scope of animals which may be treated according to the claimed invention. Some antigens are weakly immunogenic when administered alone or are toxic to the individual at concentrations which evoke

immune responses in said individual. An immune adjuvant may enhance the immune response of the individual to an antigen. The adjuvant effect may also lower the dose of said antigen effective to achieve an immune response in said individual.

The term "vaccine composition" herein refers to a composition capable of producing an immune response in an individual administered the composition. A vaccine composition, according to the invention, may produce immunity against disease in individuals or may provide therapy in individuals with established disease. The vaccine composition may modulate immune response and be a treatment of autoimmune diseases. The combination of saponin and immunostimulatory polymer of the present invention may be administered to enhance the immune response to any antigen. One of ordinary skill in the art would readily appreciate that activation of CTL activity may produce immunity against disease not only prophylactically but also therapeutically (after development of disease). Preferably, the vaccine composition stimulates Th 1 and Th 2 immunity. More preferably, the vaccine composition enhances antibody production to an antigen and enhances a cell-mediated immune response to an antigen.

The immune adjuvant composition of the invention may enhance antibody production to an antigen and enhance the cell-mediated immune response to an antigen in a positive synergistic manner. The synergistic adjuvant effect of the immunostimulatory polymeric adjuvant and the saponin adjuvant described herein may be shown in a number of ways. For example, a synergistic adjuvant effect may be demonstrated as an increase in the maximum expected immune response. One

may expect an additive effect of combining two adjuvants. Specifically, if one adjuvant, used at optimum doses, produces "X" and the other adjuvant, also used at optimum doses, produces "Y" antibody, then the combination may be expected to produce "X+Y" if the result is additive and not synergistic. A maximum level of response that is considerably higher than "X+Y" would be considered a synergistic effect and would be unexpected. A second indication of synergism would be the appearance of a substantial adjuvant effect at doses that are normally not expected to produce an adjuvant effect. A third indication of synergism would be the appearance of an immune response with earlier kinetics than expected for either adjuvant alone.

Polyorganophosphazenes, including phosphazene polyelectrolytes, may be synthesized by a macromolecular nucleophilic substitution reaction of poly(dichloro phosphazene) with a wide range of chemical reagents or mixture or reagents in accordance with methods known to those skilled in the art. Preferably, the phosphazene polyelectrolytes are made by reacting the poly(dichloro phosphazene) with an appropriate nucleophile or nucleophiles that displace chlorine. Desired proportions of hydrolyzable to non-hydrolyzable side chains in the polymer may be obtained by adjusting the quantity of the corresponding nucleophiles that are reacted with poly(dichlorophosphazene) and the reaction conditions as necessary. Preferred polyphosphazenes for immune adjuvant activity have a molecular weight of over 1,000.

For example, poly[(carboxylatophenoxy)(glycinato)phosphazene] (PC-G1PP) is prepared by the nucleophilic substitution reaction of the chlorine atoms of the

poly(dichlorophosphazene) with propyl phydroxybenzoate and ethyl glycinate hydrochloride (PC-G1PP synthesis). The poly[(aryloxy)(glycinato)phosphazene] ester thus obtained is then hydrolyzed to the corresponding poly(carboxylic acid). Other polyphosphazenes may be prepared as described by Allcock, et al., *Inorg, Chem.* 11:2584 (1972); Allcock, et al., *Macromolecules* 16:715 (1983); Allcock, et al., *Macromolecules* 19:1508 (1986); Allcock, et al., *Biomaterials* 19:500 (1988); Allcock, et al., *Macromolecules* 21:1980 (1988); Allcock, et al., *Inorg. Chem.* 21:515521 (1982); Allcock, et al., *Macromolecules* 22:7579 (1989); Allcock, et al., U.S. Patent Nos. 4,440,921; 4,495,174; 4,880,622; Margill, et al., U.S. Patent No. 4,946,938; Cohen, et al., U.S. Patent No. 5,149,543; and Grolleman, et al., *J. Controlled Release* 3:143 (1986), the teachings of which, and polymers disclosed therein, are incorporated by reference herein.

Typical antigens suitable for the enhanced immune response include antigens derived from, but not limited to, any of the following: viruses, such as feline leukemia virus, feline immunodeficiency virus, influenza, HIV-1, HIV-2, rabies, measles, herpes virus, hepatitis B virus, or hoof and mouth disease; bacteria, such as anthrax, chlamydia, diphtheria, Lyme disease, *Clostridium tetani, Neisseria gonorrhea, Streptococcus pneumoniae, Haemophilus influenza*, tetanus toxoid, or tuberculosis; protozoans, such as *Babeosis bovis* or Plasmodium; or cells. The antigen may be a protein, a glycoprotein, a peptide, a polysaccharide, a lipid, a glycolipid, a phospholipid, a ceramide, or a nucleic acid encoding an antigenic protein or peptide of interest. The antigens may be purified from a natural source, synthesized by

means of solid phase synthesis, or may be obtained by means of genetic engineering. The antigens may be inactivated whole or fractionated viruses or bacteria. The antigens may also be semisynthetic products of an antigen purified from a natural source. The antigen as defined herein elicits an immunogenic response in an individual. Preferably, such individuals are humans, however, the invention is not intended to be so limiting. Any animals that may experience the beneficial effects of the vaccines of the invention are within the scope of animals which may be treated according to the claimed invention. As defined herein, the immunogenic response may be humoral or cell mediated. In the event the material to which the immunogenic response is to be directed is poorly antigenic, it may be conjugated to a carrier such as albumin or to a hapten, using standard covalent binding techniques, for example, with one of the several commercially available reagent kits.

In one embodiment, the immunostimulatory polymeric adjuvant of the immune adjuvant composition is used to deliver a nucleic acid which encodes an antigen to a mucosal surface where the nucleic acid is expressed.

In a preferred embodiment of the immunogenic composition or vaccine, approximately one part of polymer is dissolved in 10 parts 3% Na_2CO_3 aqueous solution while stirring, then 10 to 90 parts phosphate buffer, pH 7.4, is slowly added. The saponin and antigen are then added to this mixture after the addition of the phosphate buffer.

Alternatively, polyphosphazene microspheres containing the antigen, polyphosphazene and saponin adjuvant may be prepared by simply mixing the

components in an aqueous solution, and then coagulating the polymer together with the substance by mechanical forces to form a microparticle. The microparticle/saponin may be stabilized, if necessary or desired, using electrolytes, pH changes, organic solvents, heat or frost to form polymer matrices encapsulating biological material (antigen, saponin). The polymer may also be used to encapsulate the antigen/saponin, for example, using the method of Cohen, et al., U.S. Patent No. 5,149,543, the teachings of which are incorporated herein, or by spray drying a solution of polymer, antigen, and saponin.

The polymer may also be covalently conjugated with the antigen to create a water-soluble conjugate in accordance with methods well-known to those skilled in the art, usually by covalent linkage between an amino or carboxyl group on the antigen and one of the ionizable side groups on the polymer. The saponin may be covalently conjugated to the antigen by covalent linkage between an amino or carboxyl group on the antigen and the glucuronic acid carboxyl on the saponin.

In an alternative preferred embodiment, the polymer is cross-linked with a multivalent ion, preferably using an aqueous solution containing multivalent ions of the opposite charge to those of the charged side groups of the polyphosphazene, such as multivalent cations if the polymer has acidic side groups or multivalent anions if the polymer has basic side groups.

Preferably, the polymers are cross-linked by di- and trivalent metal ions such as calcium, copper, aluminum, magnesium, strontium, barium, tin, zinc, and iron, organic cations such as poly(aminoacid)s, or other polymers such as

poly(ethyleneimine), poly(vinylamine) and polysaccharides.

It will be understood by those skilled in the art that the immunogenic vaccine composition may contain other physiologically acceptable ingredients such as water, saline, polysorbate 80, cyclodextrins, or a mineral oil such as Drakeol™, Markol™, and squalene, to form an emulsion. The effective compositions of the present invention may be employed in such forms as capsules, liquid solutions, suspensions or elixirs for oral administration, or sterile liquid forms such as solutions or suspensions. Any inert acceptable carrier may preferably be used, such as saline, PBS, polysorbate 80 in saline or PBS, cyclodextrins, or any such acceptable carrier in which the compositions of the present invention have suitable solubility properties for use of the present invention.

The immunogenic composition may be administered as a vaccine by any method known to those skilled in the art that elicits an immune response, including parenterally, orally, or by transmembrane or transmucosal administration, or other suitable means. Preferably, the vaccine is administered parenterally (intravenously, intramuscularly, subcutaneously, intraperitoneally, etc.), and preferably subcutaneously. Nonlimiting examples of routes of delivery to mucosal surfaces are intranasal (or generally, the nasal associated lymphoid tissue), oral, respiratory, vaginal, and rectal.

The dosage is determined by the antigen loading and by standard techniques for determining dosage and schedules for administration for each antigen, based on titer of antibody elicited by the polymer-antigen administration, as demonstrated by

the following examples. The dosage administered may be dependent upon the age, weight, kind of concurrent treatment, if any, and nature of the antigen administered. The initial dose may be followed up with a booster dosage after a period of about two to eight weeks, preferably about four weeks, to enhance the immunogenic response. Further booster dosages may also be administered.

Although in the preferred embodiment the vaccine composition is administered simultaneously, in an alternative embodiment, the polymer, saponin, and antigen components of the vaccine composition are administered separately to the same or nearby site. The polymer serves to attract cells of the immune system to the site, where they process the antigen.

The vaccine compositions, immune adjuvant compositions, and methods of use will be further understood by reference to the following non-limiting examples.

EXAMPLES

A well-established animal model was used to assess whether formulations of an immunostimulatory polymer and a saponin together could function as an optimized immune adjuvant. In brief, experiments were set up to compare these formulations to simple formulations of the saponin and the immunostimulatory polymer, used separately. The immunostimulatory polymer that was selected to serve as an adjuvant was polyphosphazene (PCPP). The saponin that was selected to serve as an adjuvant was QS-21.

One experiment evaluated whether PCPP in combination with QS-21 may

serve as an adjuvant for a subunit vaccine, ovalbumin (OVA), in mice in inducing CTL responses and/or antibody responses. In this experiment, an immune adjuvant consisting of PCPP with suboptimal doses of QS-21 (< 2.5 µg) was evaluated to assess whether PCPP may affect the adjuvant effect of QS-21 at these low doses.

A second experiment was performed to determine whether PCPP and QS-21 combinations could enhance antigen-specific antibody production, and/or influence the isotype profile of antigen-specific antibody response and functional antibody response to a subunit antigen, inactivated influenza virus.

A third experiment was performed to determine whether PCPP and QS-21 combinations could enhance antigen-specific antibody production and/or influence the isotype profile of antigen-specific antibody response and functional antibody response to the antigen Type 14 *S. pneumonia* capsular polysaccharide.

The experiments were done using materials from the following suppliers: OVA, Grade VI (Sigma), split inactivated influenza virus strain X-31 (Spafas, Storrs, CT), QS-21 (Aquila Biopharmaceuticals, Inc., Framingham, MA), PCPP (Avant Immunotherapeutics, Inc., Needham, MA, formerly Virus Research Institute, Cambridge, MA), Type 14 *S. pneumonia* capsular polysaccharide (ATCC, Rockville, MD).

Example 1
OVA Antigen Specific Serum IgG

C57BL/6 mice (5 per group, female, 8-10 weeks of age) were immunized by subcutaneous route at days 0, 14, and 28. The vaccines were 25 µg ovalbumin (OVA)

subcutaneous route at days 0, 14, and 28. The vaccines were 25 μg ovalbumin (OVA) (antigen) plus the indicated doses of adjuvant in a total volume of 0.2 ml phosphate-buffered saline. Serum titers to OVA were determined by ELISA on sera collected on day 42 from the mice immunized as described in above. IgG antibody titers were determined for individual mice (5 mice per group) and are plotted as a geometric mean titer (GMT). The IgG titers were highest in groups receiving QS-21 alone (at the 10 μg dose) or PCPP alone (at 100 μg dose) or 10 μg QS-21 in combination with either 10 or 100 μg PCPP as seen in Figure 2.

Example 2
OVA-Specific CTL Induced by QS-21 and PCPP

Splenocytes from mice immunized as described in Example 1 were removed at day 42 for use as effector cells in the cytotoxic T lymphocyte (CTL) assay. Splenocytes were stimulated *in vitro* for 6 days with mitomycin C-treated E.G7-OVA cells and then used in a standard ^{51}Cr release CTL assay. E.G7-OVA cells (loaded with ^{51}Cr) were used as target cells. The background lysis of EL4 cells (not transfected by OVA) was subtracted from the lysis of E.G7-OVA cells to obtain a percent (%) antigen-specific lysis.

The results, as shown in Figure 3, indicate that no lysis was observed in the absence of adjuvant, with either 10 μg or 100 μg PCPP dose, or with 1.25 μg of QS-21 (suboptimal dose). Formulations containing optimal doses of QS-21 (10 μg) induced a potent CTL response whether the QS-21 was used alone or in combination with either 10 μg or 100 μg PCPP. Surprisingly, the suboptimal dose of QS-21 in combination with 100 μg of PCPP also induced a significant CTL response.

Example 3
Influenza Antigen Specific Serum IgG Antibody
and its Subclasses IgG1 and IgG2a

Balb/C mice (5 per group, female, 6-8 weeks of age) were immunized by subcutaneous route at day 0. The vaccines were 5 µg influenza (antigen) plus the indicated doses of adjuvant in a total volume of 0.1 ml phosphate buffered saline. Serum titers to influenza were determined by ELISA on sera collected on day 21 and day 42 from the mice. IgG antibody titers as well as subclass IgG1 and IgG2a antibody titers, were determined for individual mice and are plotted as a geometric mean titer. Hemagglutination-inhibition titers were also measured.

Figure 4 shows the influenza specific serum IgG response. IgG serum antibody titers were not observed for the suboptimal doses of QS-21 (2 µg) or PCPP (10 µg) or in the unadjuvanted group. IgG responses were detectable, however, with the optimal doses of PCPP (100 µg) and QS-21 (20 µg) , as well as with the suboptimal dose of PCPP (10 µg) and QS-21 at either 2 or 20 µg. The greatest titers of IgG antibodies were detected with the 100 µg PCPP doses combined with QS-21 at either 2 or 20 µg. The IgG antibody response with either of these adjuvant combinations was synergistically enhanced compared to using either adjuvant alone. Surprisingly, the combination of 2 or 20 µg QS-21 with 100 µg PCPP induced titers that were higher than the addition of titers from the individual adjuvants.

For example, Figure 4 shows that at day 42, the GMT of the group receiving 20 µg QS-21 and 100 µg PCPP was 524,288, which was 3-fold higher than 100 µg PCPP and 8-fold higher than 20 µg QS-21. The GMT of the group receiving 100 µg PCPP

and 2 µg QS-21 was 301,124, which was 1.7-fold higher than 100 µg PCPP and 56-fold higher than 2 µg QS-21. The GMT of the group receiving 10 µg PCPP and 20 µg QS-21 was 99,334, which was 21-fold higher than 10 µg PCPP and 1.5-fold higher than 20 µg QS-21. The GMT of the group receiving 10 µg PCPP and 2 µg QS-21 was 28,526, which was 6-fold higher than 10 µg PCPP and 5-fold higher than 2 µg QS-21. These titers are higher than the simple addition of titer increases.

As evident in Figure 5, the IgG2a response was not detectable in any PCPP dose used alone, with the suboptimal QS-21 dose of 2 µg, or with the unadjuvanted group. The IgG2a response was minimally detected in the optimal QS-21 dose of 20 µg and in several of the combinations of PCPP and QS-21. Surprisingly, the IgG2a antibody response was significantly enhanced with the adjuvant combination of 20 µg QS-21 and 100 µg PCPP. This result was observed both at day 21 and day 42.

In Figure 6, the results of the IgG1 antibody responses are shown. No response was seen with the unadjuvanted group or with the suboptimal doses of QS-21 (2 µg) or PCPP (10 µg). Influenza specific serum IgG1 responses were observed for the other adjuvant doses, alone or in combination. A synergistic effect on the antibody titer may be seen for the 100 µg PCPP and 2 µg QS-21 or the 100 µg PCPP and 20 µg QS-21 combinations, compared to either component alone at day 21 post-immunization. Hence, there was an improved kinetics of immune response for IgG1 titers.

Example 4
Influenza Antigen-Specific Hemagglutination
Inhibiting Serum IgG Response

Serum titers to influenza antigen were determined by hemagglutination inhibition assay (HIA) on sera collected on day 21 and day 42 from the mice immunized in Example 3. The HIA assay is one measure of functional antibody responses against the influenza virus. The hemagglutination inhibition antibody response is shown in Figure 7. The results show that the hemagglutination inhibition titers were highest in groups receiving the adjuvant combination of 100 μg PCPP and 2 μg QS-21 or 100 μg PCPP and 20 μg QS-21. These titer levels were synergistically enhanced compared to the titer levels of either component alone. This result was observed both at day 21 and day 42.

Example 5
Streptococcal Polysaccharide Antigen-Specific
Serum IgG1, IgG2a, and IgG3 Antibody

Balb/C mice (10 per group, female, 8-10 weeks of age) were immunized by subcutaneous route at day 0. The vaccines were 0.5 μg Type 14 *S. pneumonia* capsular polysaccharide (antigen) plus the indicated doses of adjuvant in a total volume of 0.2 ml phosphate-buffered saline. Sera were collected from 5 mice per group on day 21. The remaining 5 mice per group were immunized by subcutaneous route on day 28. Sera were collected from these mice on day 42. Serum titers to polysaccharide were determined by ELISA on sera collected on day 21 (mice immunized once) and on sera collected on day 42 (mice immunized twice). IgG

subclasses IgG1, IgG2a, and IgG3 titers were determined for an equivolume serum pool.

As evident in Figures 8, 9, and 10, the combination of PCPP and QS-21 has an adjuvant effect that is higher than when either is used alone. A single immunization with polysaccharide alone (no adjuvant) yields an IgG1 titer of 5 (Figure 8). A single immunization with an optimal (10 µg) dose of QS-21 yields an IgG1 titer of 41, showing a moderate adjuvant effect. This dose of QS-21 was expected to be optimal, based on results in mice with other antigens. A single immunization with an optimal (100 µg) dose of PCPP yields an IgG1 titer of 5 (no apparent adjuvant effect). This dose of PCPP was expected to be optimal, based on results in mice with other antigens. The combination of these two doses together yields an IgG1 titer of 342 (8-fold higher than with QS-21, 68-fold higher than PCPP). The combination of these two doses together after two immunizations (day 42 sera) yields an IgG1 titer that is 19-fold higher than for QS-21 and that is 165-fold higher than for PCPP.

Figure 9 shows the results for the antigen-specific serum IgG2a response. A single dose of polysaccharide yields an IgG2a titer of 5. The addition of 10 µg of QS-21 yields a titer of 93. The addition of PCPP to the polysaccharide yields a titer of 35. However, the combination of these doses of QS-21 and PCPP yields a titer that is still 3-fold increased over QS-21 and is 9-fold increased over PCPP. Two immunizations with the combination yield a titer that is 8-fold higher than QS-21 and is 19-fold higher that PCPP.

Figure 10 shows the IgG3 data for these same sera. A single immunization

with polysaccharide yields a titer of 5. The addition of QS-21 yields a titer of 80. The addition of 100 μg PCPP yields a titer of 59. The combination of these two doses yields a titer that is 6-fold increased over QS-21 and is 8-fold increased over PCPP. Two immunizations with the combination yield a titer that is 5-fold higher than QS-21 and is 24-fold higher than for PCPP. These titers are higher than expected from the simple addition of titer increases.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications may be made thereto without departing from the spirit or scope of the invention as set forth below.

We claim:

1. A vaccine composition comprising:

(a) an antigen;

(b) one or more saponin adjuvants; and

(c) an immunostimulatory polymeric adjuvant.

2. The vaccine composition as claimed in claim 1, wherein the saponin adjuvant is derived from *Quillaja saponaria.*

3. The vaccine composition as claimed in claim 2, wherein the saponin adjuvant comprises a substantially pure saponin adjuvant.

4. The vaccine composition as claimed in claim 3, wherein the substantially pure saponin adjuvant comprises QS-7, QS-17, QS-18, QS-21, QS-21V1, or QS-21V2.

5. The vaccine composition as claimed in claim 4, wherein the substantially pure saponin adjuvant comprises QS-21.

6. The vaccine composition as claimed in claim 1, wherein the saponin adjuvant comprises a semisynthetic saponin adjuvant or a fraction thereof obtainable from an unpurified *Quillaja saponaria* extract, wherein the semisynthetic saponin or fraction thereof comprises at least one of QS-7, QS-17, QS-18, QS-21, QS-21V1, and

QS-21V2.

7. The vaccine composition as claimed in claim 6, wherein the semisynthetic saponin or fraction thereof consists of conjugation of the saponin glucuronic acid carboxyl to an antigen.

8. The vaccine composition as claimed in claim 1, wherein the immunostimulatory polymeric adjuvant comprises a polyorganophosphazene with an ionized or ionizable pendant group that contains carboxylic acid or hydroxyl moieties.

9. The vaccine composition as claimed in claim 8, wherein the immunostimulatory polymeric adjuvant comprises poly[di(carboxylatophenoxy)phosphazene].

10. The vaccine composition as claimed in claim 1, wherein the immunostimulatory polymeric adjuvant comprises a polyorganophosphazene with (i) an ionized or ionizable pendant group that contains carboxylic acid or hydroxyl moieties and (ii) a pendant group that is susceptible to hydrolysis under the conditions of use, to impart biodegradability to the polymer and wherein the pendant group that is susceptible to hydrolysis is selected from chlorine, amino acid, amino acid ester, imidazole, glycerol, and glucosyl.

11. The vaccine composition as claimed in claim 10, wherein the immunostimulatory polymeric adjuvant is selected from poly[di(carboxyaltophenoxy)phosphazene-co-di(glycinato)phosphazene-co(carboxylatophenoxy)(glycinato)phosphazene] and poly [di(carboxylatophenoxy)phosphazene-co-di(chloro)phosphazene-co-(carboxyaltophenoxy)-(chloro)phosphazene.

12. An immune adjuvant composition comprising:

(a) one or more saponin adjuvants; and

(b) an immunostimulatory polymeric adjuvant.

13. The immune adjuvant composition as claimed in claim 12, wherein the saponin adjuvant is derived from *Quillaja saponaria*.

14. The immune adjuvant composition as claimed in claim 13, wherein the saponin adjuvant comprises a substantially pure saponin adjuvant.

15. The immune adjuvant composition as claimed in claim 14, wherein the substantially pure saponin adjuvant comprises QS-7, QS-17, QS-18, QS-21, QS-21V1, or QS-21V2.

16. The immune adjuvant composition as claimed in claim 15, wherein the

substantially pure saponin adjuvant comprises QS-21.

17. The immune adjuvant composition as claimed in claim 12, wherein the substantially pure saponin adjuvant comprises a semisynthetic saponin adjuvant or a fraction thereof obtainable from an unpurified *Quillaja saponaria* extract, wherein the semisynthetic saponin or fraction thereof comprises at least one of QS-7, QS-17, QS-18, QS-21, QS-21V1, and QS-21V2.

18. The immune adjuvant composition as claimed in claim 17, wherein the semisynthetic saponin or fraction thereof consists of conjugation of the saponin glucuronic acid carboxyl to an antigen.

19. The immune adjuvant composition as claimed in claim 12, wherein the immunostimulatory polymeric adjuvant comprises a polyorganophosphazene with an ionized or ionizable pendant group that contains carboxylic acid or hydroxyl moieties.

20. The immune adjuvant composition as claimed in claim 19, wherein the immunostimulatory polymeric adjuvant comprises poly[di(carboxylatophenoxy)phosphazene].

21. The immune adjuvant composition as claimed in claim 12, wherein the

immunostimulatory polymeric adjuvant comprises a polyorganophosphazene with (i) an ionized or ionizable pendant group that contains carboxylic acid or hydroxyl moieties and (ii) a pendant group that is susceptible to hydrolysis under the conditions of use, to impart biodegradability to the polymer and wherein the pendant group that is susceptible to hydrolysis is selected from chlorine, amino acid, amino acid ester, imidazole, glycerol, and glucosyl.

22. The immune adjuvant composition as claimed in claim 21, wherein the immunostimulatory polymeric adjuvant is selected from poly[di(carboxyaltophenoxy)phosphazene-co-di(glycinato)phosphazene-co(carboxylatophenoxy)(glycinato)phosphazene] and poly [di(carboxylatophenoxy)phosphazene-co-di(chloro)phosphazene-co-(carboxyaltophenoxy)-(chloro)phosphazene.

23. An immune adjuvant composition comprising:

(a) one or more saponin adjuvants; and

(b) an immunostimulatory polymeric adjuvant;

wherein the composition is capable of enhancing antibody production to an antigen.

24. The immune adjuvant composition as claimed in claim 23, wherein the saponin adjuvant is derived from *Quillaja saponaria*.

25. The immune adjuvant composition as claimed in claim 24, wherein the saponin adjuvant comprises a substantially pure saponin adjuvant.

26. The immune adjuvant composition as claimed in claim 25, wherein the substantially pure saponin adjuvant comprises QS-7, QS-17, QS-18, QS-21, QS-21V1, or QS-21V2.

27. The immune adjuvant composition as claimed in claim 26, wherein the substantially pure saponin adjuvant comprises QS-21.

28. The immune adjuvant composition as claimed in claim 23, wherein the substantially pure saponin adjuvant comprises a semisynthetic saponin adjuvant or a fraction thereof obtainable from an unpurified *Quillaja saponaria* extract, wherein the semisynthetic saponin or fraction thereof comprises at least one of QS-7, QS-17, QS-18, QS-21, QS-21V1, and QS-21V2.

29. The immune adjuvant composition as claimed in claim 28, wherein the chemical modification of the saponin or fraction thereof consists of conjugation of the saponin glucuronic acid carboxyl to an antigen.

30. The immune adjuvant composition as claimed in claim 23, wherein the immunostimulatory polymeric adjuvant comprises a polyorganophosphazene with

an ionized or ionizable pendant group that contains carboxylic acid or hydroxyl moieties.

31. The immune adjuvant composition as claimed in claim 30, wherein the immunostimulatory polymeric adjuvant comprises poly[di(carboxylatophenoxy)phosphazene].

32. The immune adjuvant composition as claimed in claim 23, wherein the immunostimulatory polymeric adjuvant comprises a polyorganophosphazene with (i) an ionized or ionizable pendant group that contains carboxylic acid or hydroxyl moieties and (ii) a pendant group that is susceptible to hydrolysis under the conditions of use, to impart biodegradability to the polymer and wherein the pendant group that is susceptible to hydrolysis is selected from chlorine, amino acid, amino acid ester, imidazole, glycerol, and glucosyl.

33. The immune adjuvant composition as claimed in claim 32, wherein the immunostimulatory polymeric adjuvant is selected from poly[di(carboxyaltophenoxy)phosphazene-co-di(glycinato)phosphazene-co(carboxylatophenoxy)(glycinato)phosphazene] and poly [di(carboxylatophenoxy)phosphazene-co-di(chloro)phosphazene-co-(carboxyaltophenoxy)-(chloro)phosphazene.

34. An immune adjuvant composition comprising:

(a) one or more saponin adjuvants; and

(b) an immunostimulatory polymeric adjuvant;

wherein the composition is capable of enhancing cell-mediated immunity to an antigen.

35. The immune adjuvant composition as claimed in claim 34, wherein the saponin adjuvant is derived from *Quillaja saponaria.*

36. The immune adjuvant composition as claimed in claim 35, wherein the saponin adjuvant comprises a substantially pure saponin adjuvant.

37. The immune adjuvant composition as claimed in claim 34, wherein the composition is capable of enhancing cell-mediated immunity to an antigen in a positive, synergistic manner.

38. The immune adjuvant composition as claimed in claim 36, wherein the substantially pure saponin adjuvant comprises QS-7, QS-17, QS-18, QS-21, QS-21V1, or QS-21V2.

39. The immune adjuvant composition as claimed in claim 38, wherein the substantially pure saponin adjuvant comprises QS-21.

40. The immune adjuvant composition as claimed in claim 34, wherein the substantially pure saponin adjuvant comprises a semisynthetic saponin adjuvant or a fraction thereof obtainable from an unpurified *Quillaja saponaria* extract, wherein the semisynthetic saponin or fraction thereof comprises at least one of QS-7, QS-17, QS-18, QS-21, QS-21V1, and QS-21V2.

41. The immune adjuvant composition as claimed in claim 40, wherein the semisynthetic saponin or fraction thereof consists of conjugation of the saponin glucuronic acid carboxyl to an antigen.

42. The immune adjuvant composition as claimed in claim 34, wherein the immunostimulatory polymeric adjuvant comprises a polyorganophosphazene with an ionized or ionizable pendant group that contains carboxylic acid or hydroxyl moieties.

43. The immune adjuvant composition as claimed in claim 42, wherein the immunostimulatory polymeric adjuvant comprises poly[di(carboxylatophenoxy)phosphazene].

44. The immune adjuvant composition as claimed in claim 34, wherein the immunostimulatory polymeric adjuvant comprises a polyorganophosphazene with (i) an ionized or ionizable pendant group that contains carboxylic acid or hydroxyl

moieties and (ii) a pendant group that is susceptible to hydrolysis under the conditions of use, to impart biodegradability to the polymer and wherein the pendant group that is susceptible to hydrolysis is selected from chlorine, amino acid, amino acid ester, imidazole, glycerol, and glucosyl.

45. The immune adjuvant composition as claimed in claim 44, wherein the immunostimulatory polymeric adjuvant is selected from poly[di(carboxyaltophenoxy)phosphazene-co-di(glycinato)phosphazene-co(carboxylatophenoxy)(glycinato)phosphazene] and poly [di(carboxylatophenoxy)phosphazene-co-di(chloro)phosphazene-co-(carboxyaltophenoxy)-(chloro)phosphazene.

46. A method for stimulating immunity to an antigen in an individual comprising administering an effective amount of a vaccine composition according to claim 1.

47. The method as claimed in claim 46, wherein the saponin adjuvant is derived from *Quillaja saponaria.*

48. The method as claimed in claim 47, wherein the saponin adjuvant comprises a substantially pure saponin adjuvant.

49. The method as claimed in claim 48, wherein the substantially pure saponin adjuvant comprises QS-7, QS-17, QS-18, QS-21, QS-21V1, or QS-21V2.

50. The method as claimed in claim 49, wherein the substantially pure saponin adjuvant comprises QS-21.

51. The method as claimed in claim 46, wherein the substantially pure saponin adjuvant comprises a semisynthetic saponin adjuvant or a fraction thereof obtainable from an unpurified *Quillaja saponaria* extract, wherein the semisynthetic saponin or fraction thereof comprises at least one of QS-7, QS-17, QS-18, QS-21, QS-21V1, and QS-21V2.

52. The method as claimed in claim 51, wherein the semisynthetic saponin or fraction thereof consists of conjugation of the saponin glucuronic acid to carboxyl to an antigen.

53. The method as claimed in claim 46, wherein the immunostimulatory polymeric adjuvant comprises a polyorganophosphazene with an ionized or ionizable pendant group that contains carboxylic acid or hydroxyl moieties.

54. The method as claimed in claim 53, wherein the immunostimulatory polymeric adjuvant comprises poly[di(carboxylatophenoxy)phosphazene].

55. The method as claimed in claim 46, wherein the immunostimulatory polymeric adjuvant comprises a polyorganophosphazene with (i) an ionized or ionizable pendant group that contains carboxylic acid or hydroxyl moieties and (ii) a pendant group that is susceptible to hydrolysis under the conditions of use, to impart biodegradability to the polymer and wherein the pendant group that is susceptible to hydrolysis is selected from chlorine, amino acid, amino acid ester, imidazole, glycerol, and glucosyl.

56. The method as claimed in claim 55, wherein the immunostimulatory polymeric adjuvant is selected from poly[di(carboxyatophenoxy)phosphazene-co-di(glycinato)phosphazene-co(carboxylatophenoxy)(glycinato)phosphazene] and poly [di(carboxylatophenoxy)phosphazene-co-di(chloro)phosphazene-co-(carboxyaltophenoxy)-(chloro)phosphazene.

57. A method for enhancing antibody production to an antigen in an individual comprising administering an effective amount of an immune adjuvant composition according to claim 12.

58. A method for further enhancing antibody production to an antigen in positive, synergistic manner in an individual comprising administering an effective amount of an immune adjuvant composition according to claim 12.

59. The method as claimed in claim 57, wherein the substantially pure saponin adjuvant comprises QS-7, QS-17, QS-18, QS-21, QS-21V1, or QS-21V2.

60. The method as claimed in claim 59, wherein the substantially pure saponin adjuvant comprises QS-21.

61. The method as claimed in claim 57, wherein the substantially pure saponin adjuvant comprises a semisynthetic saponin adjuvant or a fraction thereof obtainable from an unpurified *Quillaja saponaria* extract, wherein the semisynthetic saponin or fraction thereof comprises at least one of QS-7, QS-17, QS-18, QS-21, QS-21V1, and QS-21V2.

62. The method as claimed in claim 61, wherein the semisynthetic saponin or fraction thereof consists of conjugation of the saponin glucuronic acid carboxyl to an antigen.

63. The method as claimed in claim 57, wherein the immunostimulatory polymeric adjuvant comprises a polyorganophosphazene with an ionized or ionizable pendant group that contains carboxylic acid or hydroxyl moieties.

64. The method as claimed in claim 63, wherein the immunostimulatory polymeric adjuvant comprises poly[di(carboxylatophenoxy)phosphazene].

65. The method as claimed in claim 57, wherein the immunostimulatory polymeric adjuvant comprises a polyorganophosphazene with (i) an ionized or ionizable pendant group that contains carboxylic acid or hydroxyl moieties and (ii) a pendant group that is susceptible to hydrolysis under the conditions of use, to impart biodegradability to the polymer and wherein the pendant group that is susceptible to hydrolysis is selected from chlorine, amino acid, amino acid ester, imidazole, glycerol, and glucosyl.

66. The method as claimed in claim 65, wherein the immunostimulatory polymeric adjuvant is selected from poly[di(carboxyaltophenoxy)phosphazene-co-di(glycinato)phosphazene-co(carboxylatophenoxy)(glycinato)phosphazene] and poly [di(carboxylatophenoxy)phosphazene-co-di(chloro)phosphazene-co-(carboxyaltophenoxy)-(chloro)phosphazene.

67. A method for enhancing induction of a cell-mediated immune response to an antigen in an individual comprising administering an effective amount of an immune adjuvant composition according to claim 12.

68. A method for further enhancing induction of a cell-mediated immune response to an antigen in a positive, synergistic manner in an individual comprising administering an effective amount of an immune adjuvant composition according to claim 12.

69. The method as claimed in claim 67, wherein the substantially pure saponin adjuvant comprises QS-7, QS-17, QS-18, QS-21, QS-21V1, or QS-21V2.

70. The method as claimed in claim 69, wherein the substantially pure saponin adjuvant comprises QS-21.

71. The method as claimed in claim 67, wherein the substantially pure saponin adjuvant comprises a semisynthetic saponin adjuvant or a fraction thereof obtainable from an unpurified *Quillaja saponaria* extract, wherein the semisynthetic saponin or fraction thereof comprises at least one of QS-7, QS-17, QS-18, QS-21, QS-21V1, and QS-21V2.

72. The method as claimed in claim 71, wherein the semisynthetic saponin or fraction thereof consists of conjugation of the saponin glucuronic acid carboxyl to an antigen.

73. The method as claimed in claim 67, wherein the immunostimulatory polymeric adjuvant comprises a polyorganophosphazene with an ionized or ionizable pendant group that contains carboxylic acid or hydroxyl moieties.

74. The method as claimed in claim 73, wherein the immunostimulatory polymeric adjuvant comprises poly[di(carboxylatophenoxy)phosphazene].

75. The method as claimed in claim 67, wherein the immunostimulatory polymeric adjuvant comprises a polyorganophosphazene with (i) an ionized or ionizable pendant group that contains carboxylic acid or hydroxyl moieties and (ii) a pendant group that is susceptible to hydrolysis under the conditions of use, to impart biodegradability to the polymer and wherein the pendant group that is susceptible to hydrolysis is selected from chlorine, amino acid, amino acid ester, imidazole, glycerol, and glucosyl.

76. The method composition as claimed in claim 75, wherein the immunostimulatory polymeric adjuvant is selected from poly[di(carboxyaltophenoxy)phosphazene-co-di(glycinato)phosphazene-co(carboxylatophenoxy)(glycinato)phosphazene] and poly [di(carboxylatophenoxy)phosphazene-co-di(chloro)phosphazene-co-(carboxyaltophenoxy)-(chloro)phosphazene.

77. The immune adjuvant composition as claimed in claim 24, wherein the composition is capable of enhancing antibody production to an antigen in a positive, synergistic manner.

STRUCTURE OF QS-21

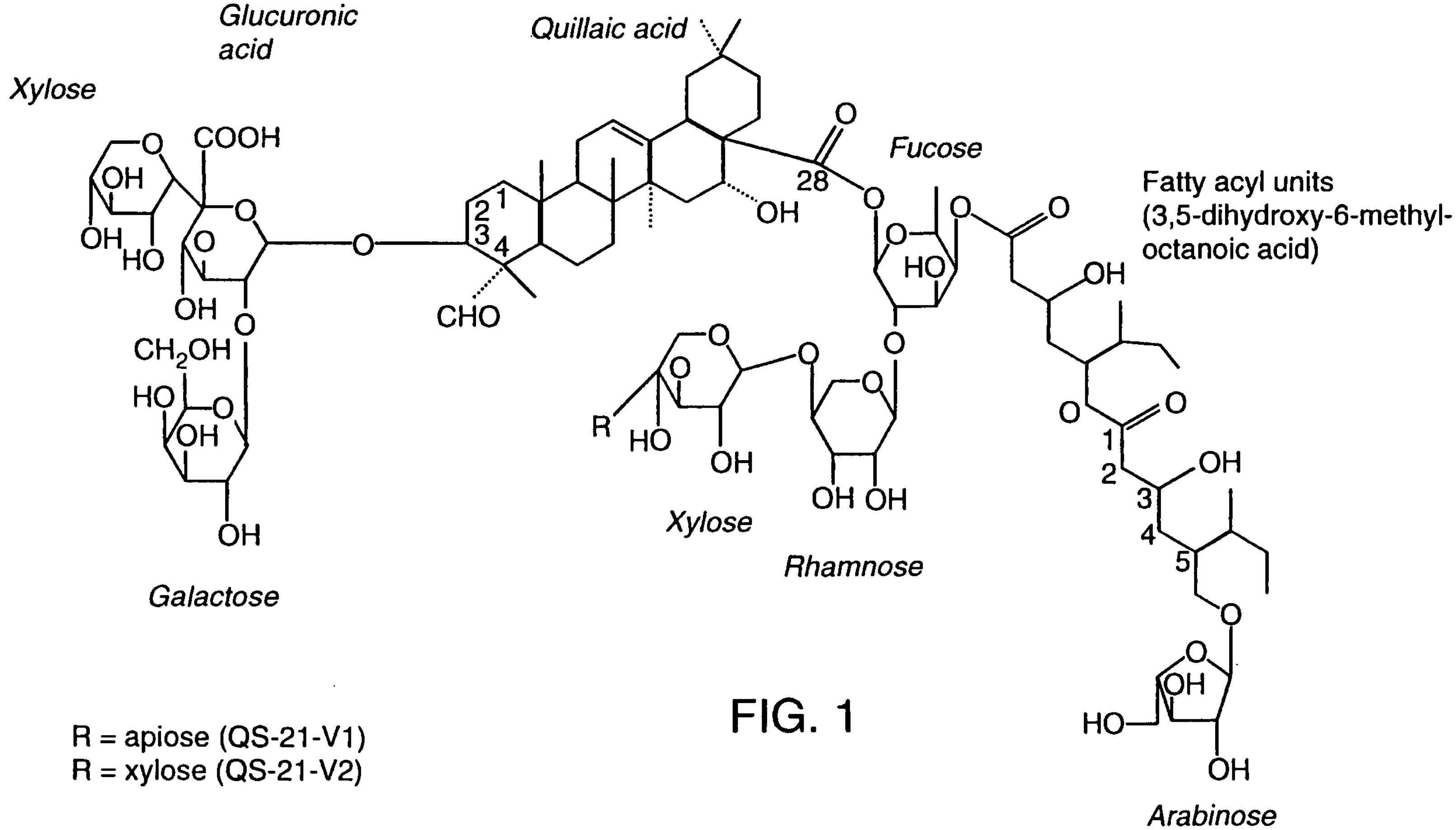


FIG. 1

R = apiose (QS-21-V1)
R = xylose (QS-21-V2)

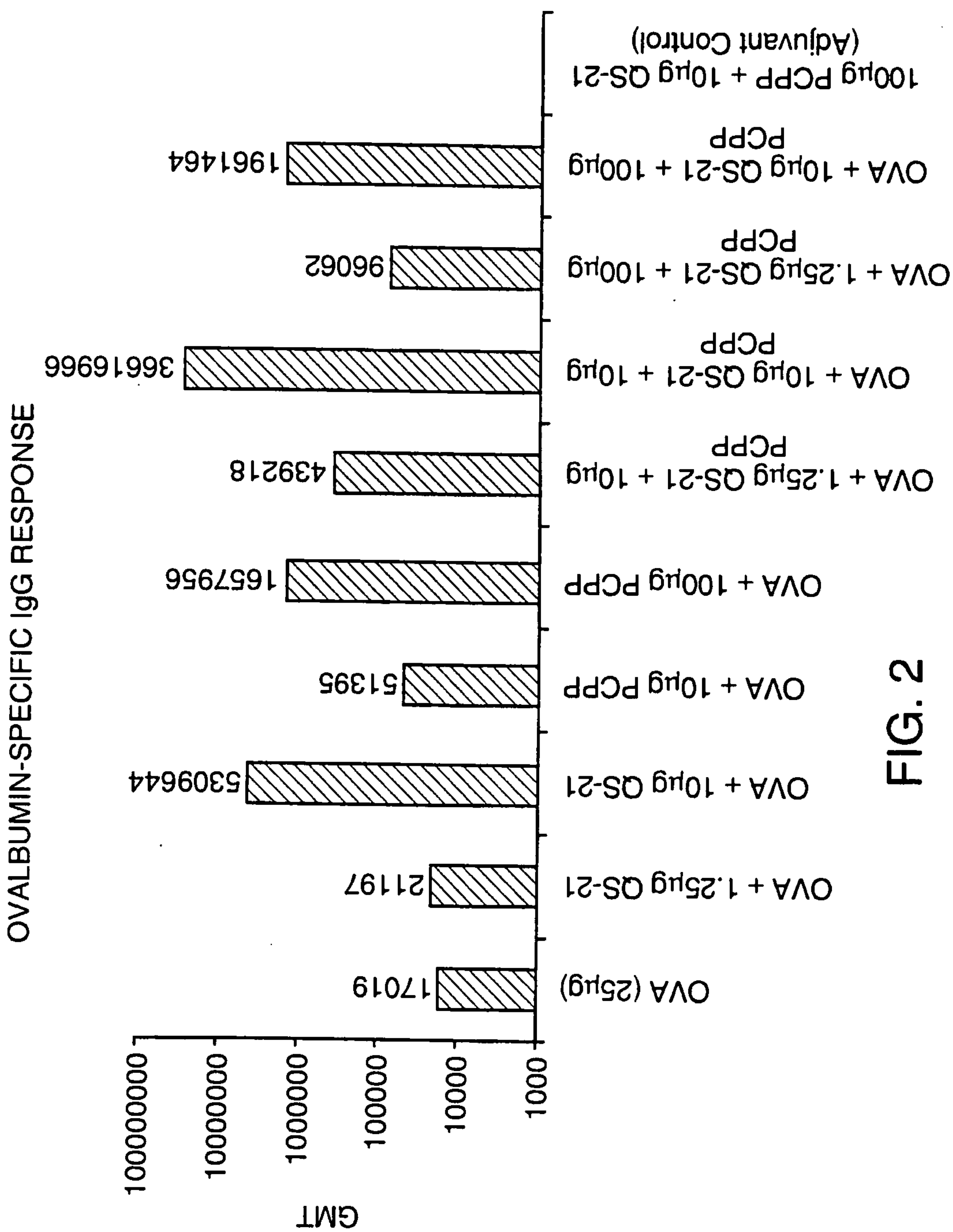

OVALBUMIN-SPECIFIC IgG RESPONSE
GMT
100000000
10000000
1000000
100000
10000
1000
OVA (25µg)
17019
OVA + 1.25µg QS-21
21197
OVA + 10µg QS-21
5309644
OVA + 10µg PCPP
51395
OVA + 100µg PCPP
1657956
OVA + 1.25µg QS-21 + 10µg PCPP
439218
OVA + 10µg QS-21 + 10µg PCPP
36616966
OVA + 1.25µg QS-21 + 100µg PCPP
96062
OVA + 10µg QS-21 + 100µg PCPP
1961464
100µg PCPP + 10µg QS-21 (Adjuvant Control)

FIG. 2

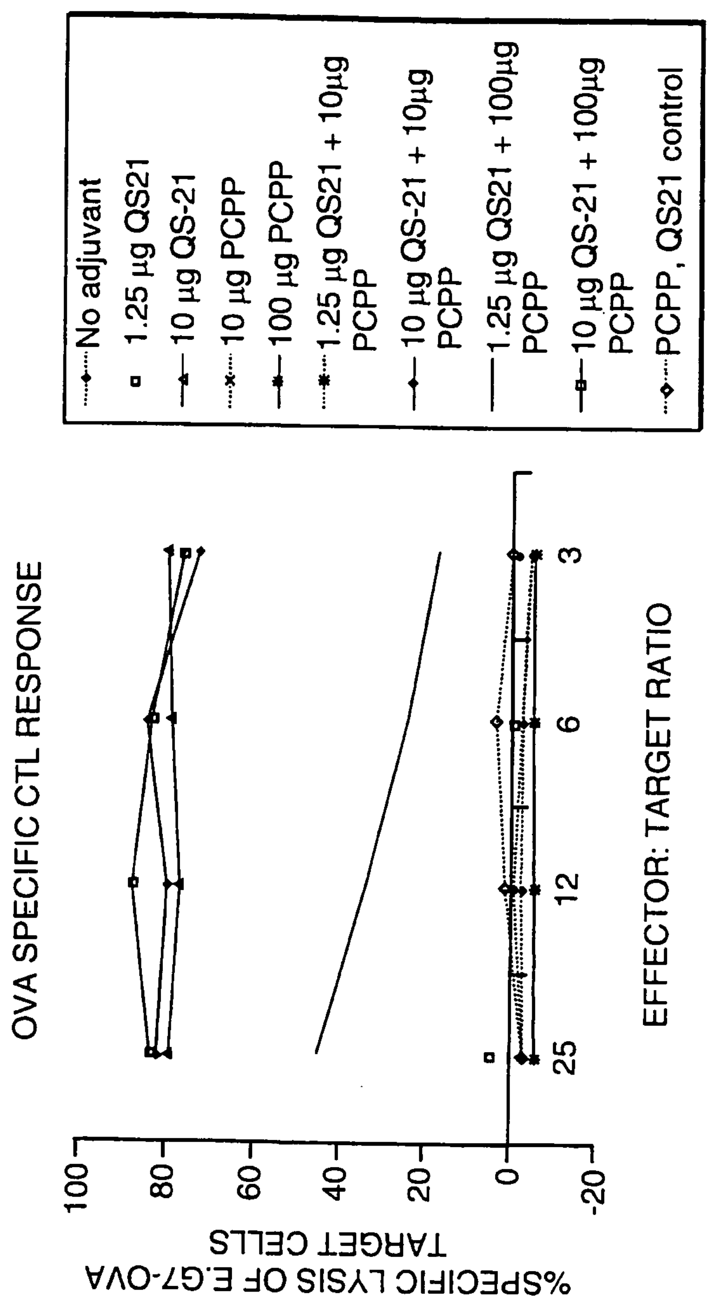

OVA SPECIFIC CTL RESPONSE
%SPECIFIC LYSIS OF E.G7-OVA TARGET CELLS
100
80
60
40
20
0
-20
25
12
6
3
EFFECTOR: TARGET RATIO
No adjuvant
1.25 µg QS21
10 µg QS-21
10 µg PCPP
100 µg PCPP
1.25 µg QS21 + 10µg PCPP
10 µg QS-21 + 10µg PCPP
1.25 µg QS21 + 100µg PCPP
10 µg QS-21 + 100µg PCPP
PCPP, QS21 control

FIG. 3

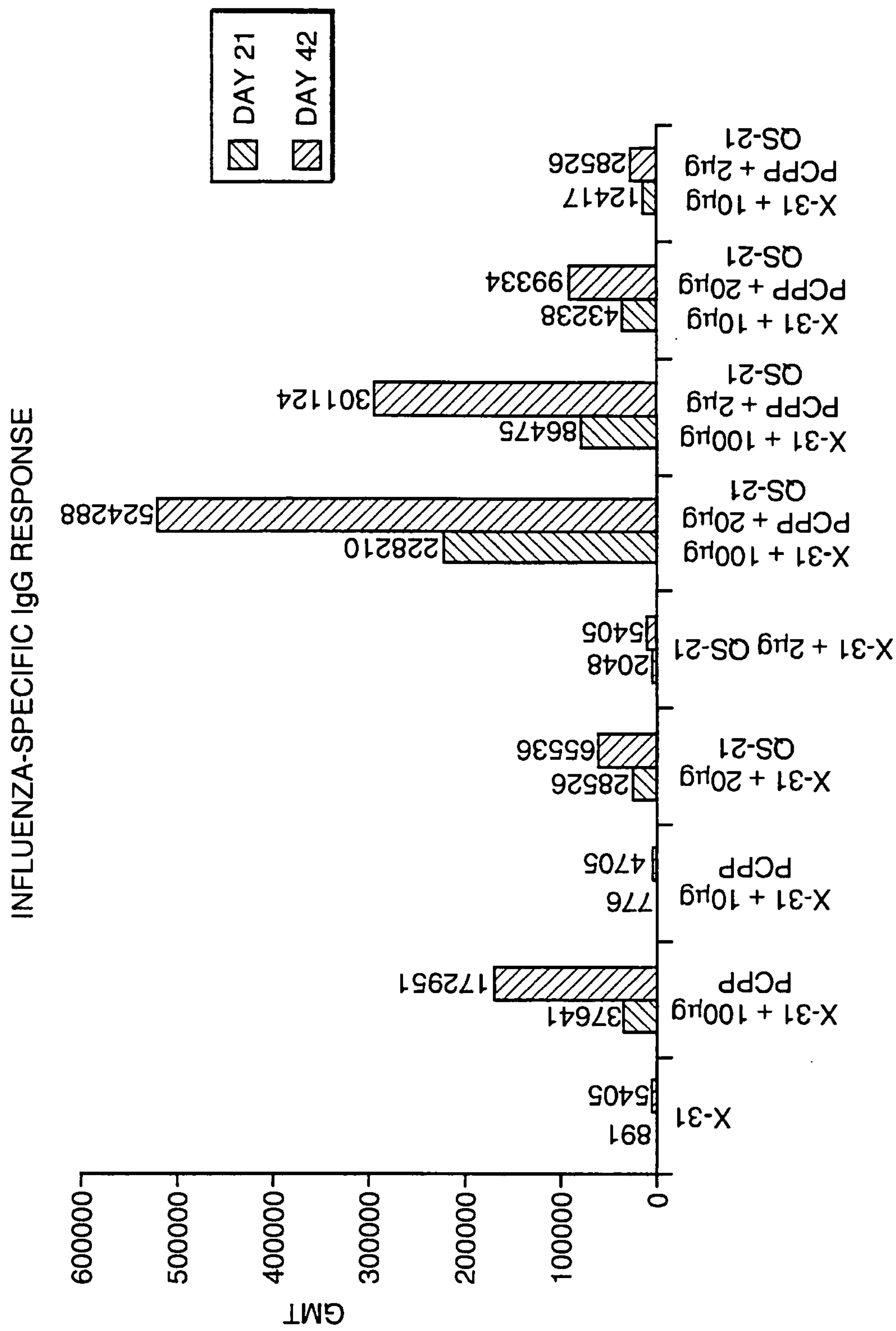

INFLUENZA-SPECIFIC IgG RESPONSE
DAY 21
DAY 42
GMT
600000
500000
400000
300000
200000
100000
0
X-31
891
5405
X-31 + 100µg PCPP
37641
172951
X-31 + 10µg PCPP
776
4705
X-31 + 20µg QS-21
28526
65536
X-31 + 2µg QS-21
2048
5405
X-31 + 100µg PCPP + 20µg QS-21
228210
524288
X-31 + 100µg PCPP + 2µg QS-21
86475
301124
X-31 + 10µg PCPP + 20µg QS-21
43238
99334
X-31 + 10µg PCPP + 2µg QS-21
12417
28526

FIG. 4

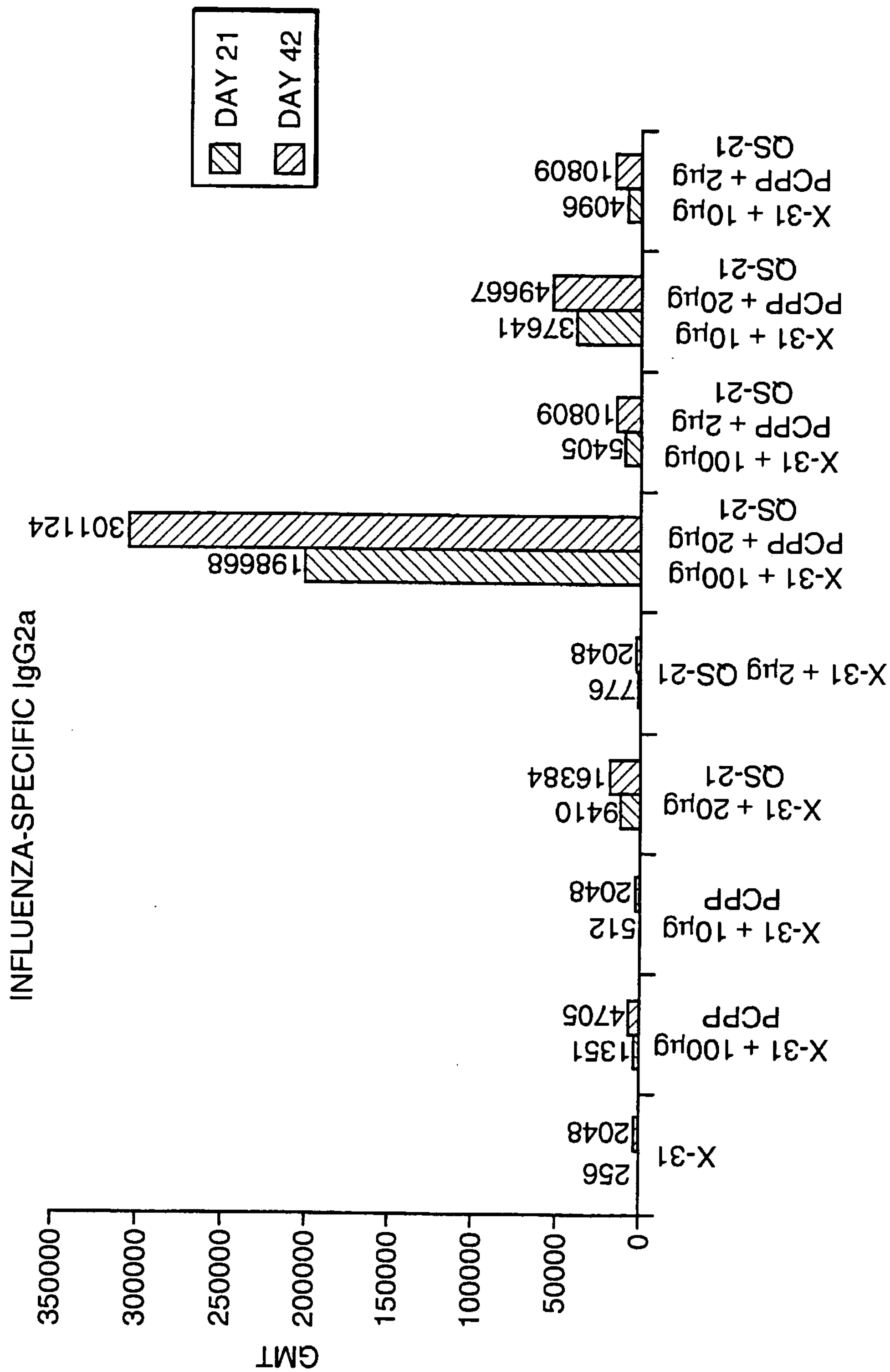

INFLUENZA-SPECIFIC IgG2a
DAY 21
DAY 42
GMT
350000
300000
250000
200000
150000
100000
50000
0
X-31
256
2048
X-31 + 100μg PCPP
1351
4705
X-31 + 10μg PCPP
512
2048
X-31 + 20μg QS-21
9410
16384
X-31 + 2μg QS-21
776
2048
X-31 + 100μg PCPP + 20μg QS-21
198668
301124
X-31 + 100μg PCPP + 2μg QS-21
5405
10809
X-31 + 10μg PCPP + 20μg QS-21
37641
49667
X-31 + 10μg PCPP + 2μg QS-21
4096
10809

FIG. 5

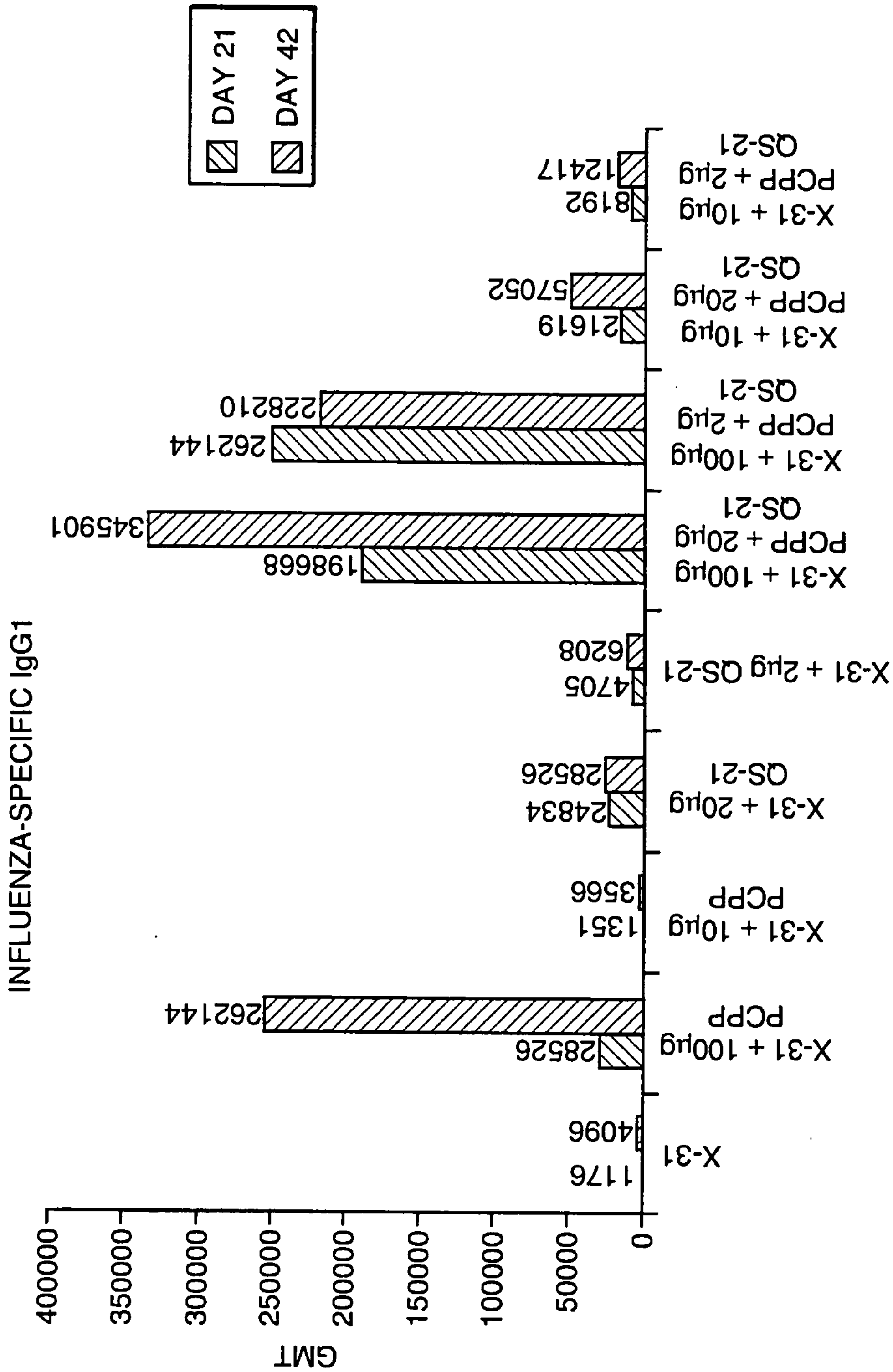

INFLUENZA-SPECIFIC IgG1
DAY 21
DAY 42
GMT
400000
350000
300000
250000
200000
150000
100000
50000
0
X-31
1176
4096
X-31 + 100µg PCPP
28526
262144
X-31 + 10µg PCPP
1351
3566
X-31 + 20µg QS-21
24834
28526
X-31 + 2µg QS-21
4705
6208
X-31 + 100µg PCPP + 20µg QS-21
198668
345901
X-31 + 100µg PCPP + 2µg QS-21
262144
228210
X-31 + 10µg PCPP + 20µg QS-21
21619
57052
X-31 + 10µg PCPP + 2µg QS-21
8192
12417

FIG. 6

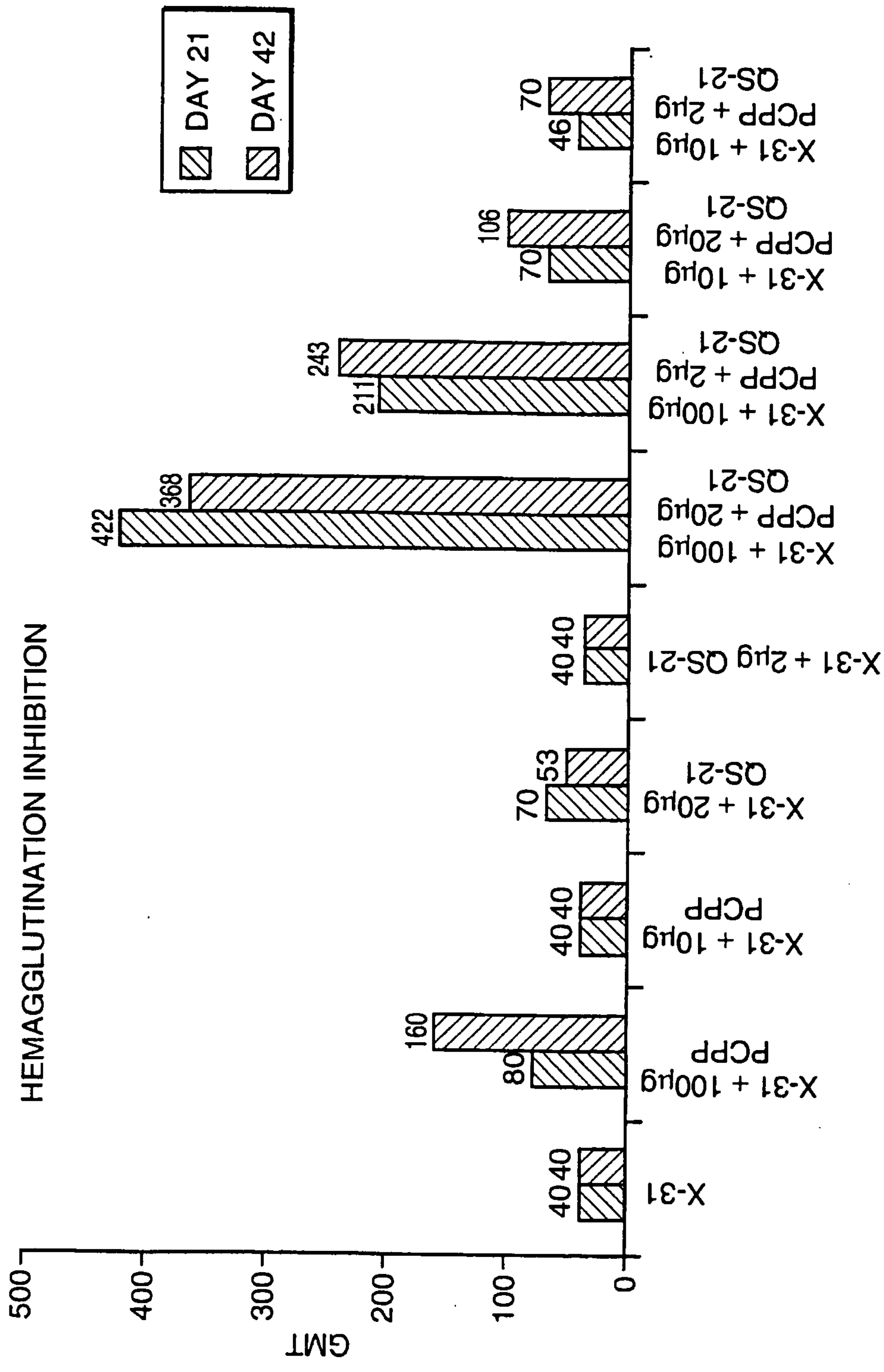

HEMAGGLUTINATION INHIBITION
DAY 21
DAY 42
GMT
500
400
300
200
100
0
X-31
40 40
X-31 + 100µg PCPP
80 160
X-31 + 10µg PCPP
40 40
X-31 + 20µg QS-21
70 53
X-31 + 2µg QS-21
40 40
X-31 + 100µg PCPP + 20µg QS-21
422 368
X-31 + 100µg PCPP + 2µg QS-21
211 243
X-31 + 10µg PCPP + 20µg QS-21
70 106
X-31 + 10µg PCPP + 2µg QS-21
46 70

FIG. 7

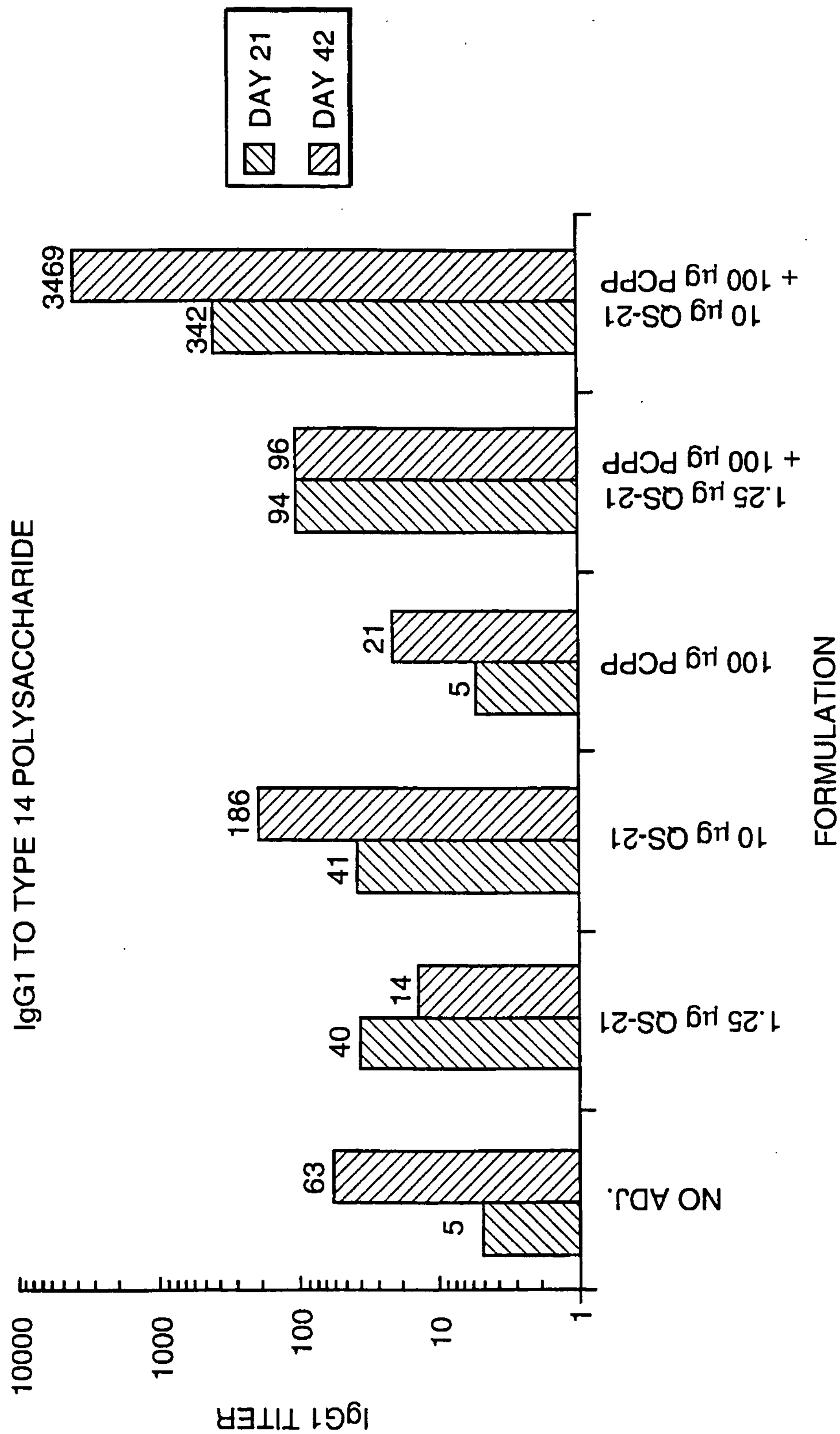

IgG1 TO TYPE 14 POLYSACCHARIDE
DAY 21
DAY 42
IgG1 TITER
10000
1000
100
10
1
5
63
40
14
41
186
5
21
94
96
342
3469
NO ADJ.
1.25 μg QS-21
10 μg QS-21
100 μg PCPP
1.25 μg QS-21 + 100 μg PCPP
10 μg QS-21 + 100 μg PCPP
FORMULATION

FIG. 8

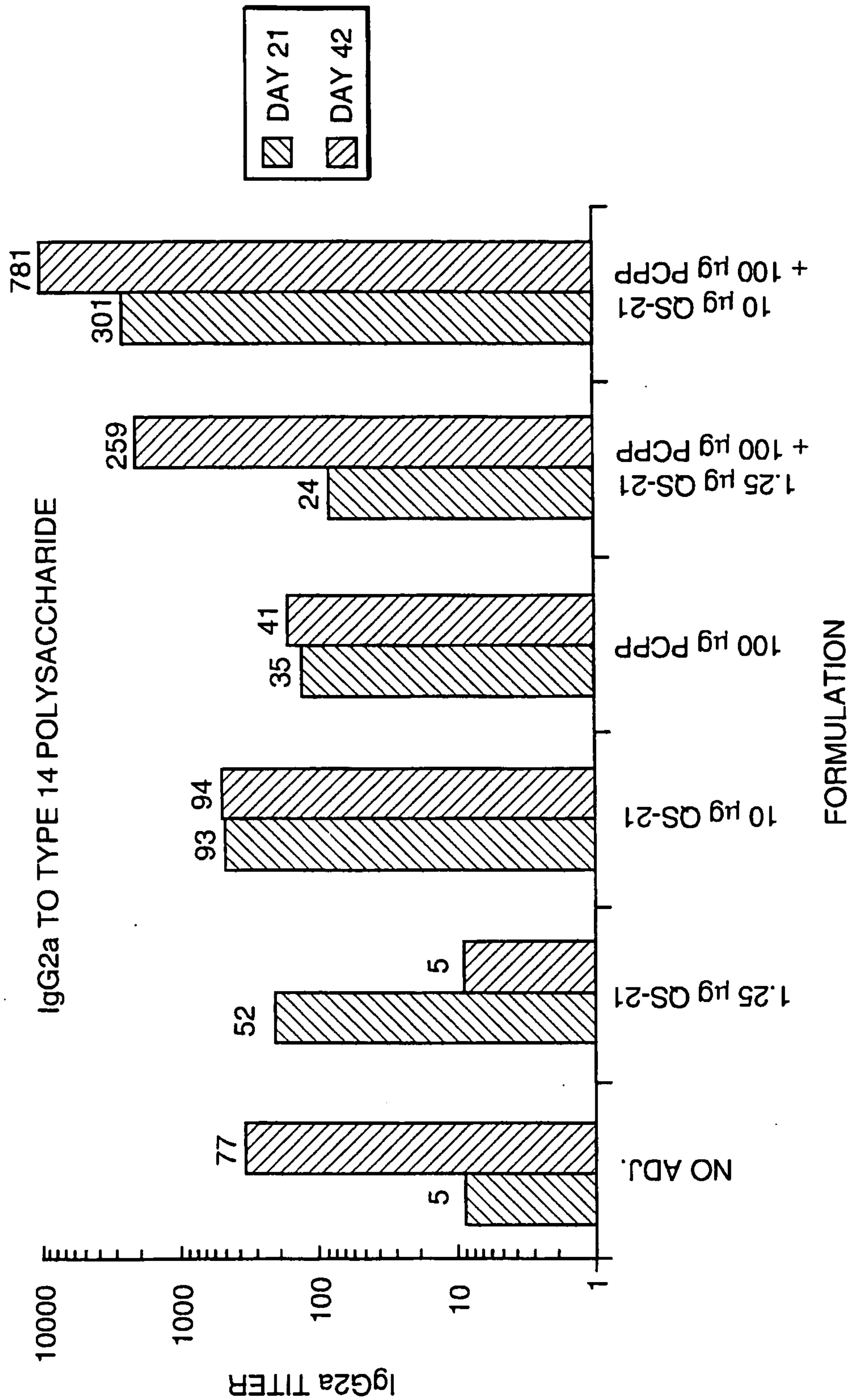

IgG2a TO TYPE 14 POLYSACCHARIDE
DAY 21
DAY 42
IgG2a TITER
10000
1000
100
10
1
NO ADJ.
1.25 µg QS-21
10 µg QS-21
100 µg PCPP
1.25 µg QS-21 + 100 µg PCPP
10 µg QS-21 + 100 µg PCPP
5
77
52
5
93
94
35
41
24
259
301
781
FORMULATION

FIG. 9

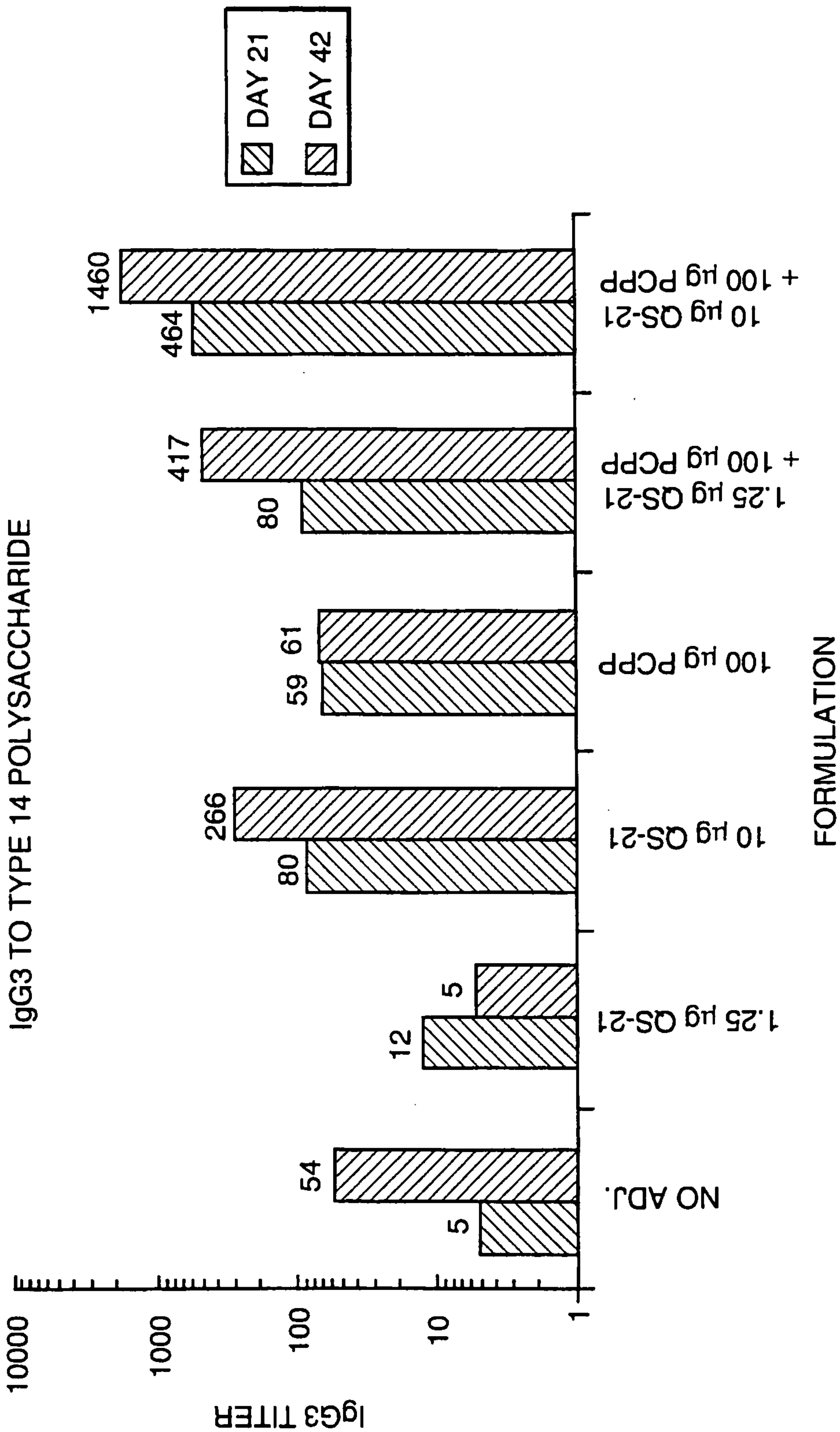

IgG3 TO TYPE 14 POLYSACCHARIDE
DAY 21
DAY 42
IgG3 TITER
10000
1000
100
10
1
5
54
12
5
80
266
59
61
80
417
464
1460
NO ADJ.
1.25 µg QS-21
10 µg QS-21
100 µg PCPP
1.25 µg QS-21 + 100 µg PCPP
10 µg QS-21 + 100 µg PCPP
FORMULATION

FIG. 10